Using the zebrafish model to understand the effects of inflammation on behaviour

A thesis submitted for the degree of

Doctor of Philosophy

by

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Statement of originality

I, Inês Moreira, declare that this thesis has been composed by myself and that the work has not been submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where stated otherwise by reference or acknowledgement. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

Section 3.3.2 in Chapter 3 contains work conducted in collaboration with the Computational Biology Facility at Liverpool University. In this section, the drug selection was performed by me, while the method described was developed by Arturas Grauslys (Computational Biology Facility at Liverpool University), as were the data extraction from LINCS L1000 and Reactome and figures F.1 to F.19. All other methods, tables and figures, and data analysis and interpretation are entirely my own work.

Abstract

The relationship between the brain, immune system, and inflammation is a complex and vital aspect of maintaining brain health throughout one's lifespan. Peripheral immune responses have been shown to play a significant role in the pathophysiology of many neuropsychiatric disorders, including depression, which represents a significant cause of disability worldwide. Given that current antidepressant medications have limited efficacy in achieving full remission for many patients, there is a need for the development of new biomarkers and treatments. Understanding the causal link between inflammatory responses and disease outcomes is crucial for the development of effective treatments for mental health disorders associated with chronic inflammation.

While rodent models have been valuable in shedding light on the immunological and mechanistic aspects of this relationship, animal behaviour is highly variable, and many animals are needed to investigate this complex biological phenomenon, carrying high ethical and financial costs. This thesis aimed to investigate the hypothesis that inflammation can disrupt zebrafish (*Danio rerio*) larvae behaviour and that the inflammation-behaviour relationship observed in mammalian models is also present in zebrafish.

While the causal relationship between immune dysfunction and the pathophysiology of depression remains unclear, antidepressants have been shown to affect both immune cells and signalling molecules, leading to both immunomodulatory and pro/anti-inflammatory effects. In this project, it's also hypothesized that the therapeutic effects of antidepressants may be partially attributed to their immunomodulatory properties.

To address these hypotheses, experimental and computational were integrated. Zebrafish were exposed to proinflammatory stimuli and *in vivo* imaging techniques

were used to investigate the effects of various treatments on the trafficking of two innate immune cells. These data were linked to the behaviour displayed by zebrafish larvae using automated tracking software. To understand the immune-modulating properties of antidepressants large-scale transcriptomic datasets were used, and the computational observations *in vivo* were validated using the zebrafish inflammation model developed in this project.

This thesis contributes to a growing body of research that explores the relationship between inflammation, behaviour and mental health disorders. These findings support the widely reported hypothesis that inflammation is associated with behavioural alterations and that antidepressant drugs may exert their effects, at least in part, through modulating immune pathways. The zebrafish model developed in this thesis is a valuable tool for investigating this relationship and for identifying potential therapeutic targets. Furthermore, this work also revealed that different classes of antidepressants interact with various immune pathways, and even within the same class, immunomodulatory action can vary, demonstrating the potential of drug-induced perturbation databases for computational drug discovery approaches. However, there are still many unanswered questions, and further studies are necessary to validate the findings presented. This thesis serves as a stepping stone for future studies aimed at elucidating the precise mechanisms underlying the relationship between inflammation and behaviour, and for identifying new therapeutic targets for mental health disorders.

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Contents

List of Abbreviations

βc β common.

5-HT 5-hydroxytryptamine (serotonin).

ACTH adrenocorticotropic hormone.

Akt protein kinase B.

AVP arginine vasopressin.

BBB blood-brain barrier.

BCR B-cell receptor.

BCSFB blood-cerebrospinal fluid barrier.

BH4 tetrahydrobiopterin.

CAAX cysteine-aliphatic-aliphatic-any amino acid.

CAMs cell adhesion molecules.

CBT cognitive behavioral therapy.

CD cluster of differentiation.

CIT citalopram.

cMAP Connectivity Map.

CNS central nervous system.

COX cyclooxygenase.

CRF corticotropin-releasing factor.

CRH corticotropin-releasing hormone.

CRP C-reactive protein.

CSF cerebrospinal fluid.

CTD comparative Toxicogenomics Database.

$CuSO_4$ copper (II) sulfate.

dpf days post-fertilization.

DSP dexamethasone sodium phosphate.

DSS dextran sodium sulfate.

ERK extracellular signal-regulated kinase.

FCERI high-affinity IgE receptor.

FDR false discovery rate.

FOXO3 forkhead box O3.

GCH-1 GTP cyclohydrolase 1.

GM-CSF granulocyte-macrophage colony-stimulating factor.

GR glucocorticoid receptor.

HFD high-fat diet.

HPA hypothalamic-pituitary-adrenal.

hpf hours post fertilization.

IBD inflammatory bowel disease.

ICAM-1 intercellular adhesion molecule 1.

IDO indoleamine 2,3-dioxygenase.

IFN interferon.

IgE immunoglobulin E.

IHME Institute for Health Metrics and Evaluation.

IL interleukin.

iNOS inducible nitric oxide synthase.

IPA ingenuity Pathway Analysis.

IRF interferon regulatory factor.

ISF interstitial fluid.

JNK c-Jun N-terminal kinase.

L-DOPA levodopa.

LINCS Library of Integrated Network-Based Cellular Signatures.

LOEC Lowest Observed Effect Concentration.

LPS lipopolysaccharide.

MAPK mitogen-activated protein kinase.

M-CSF macrophage colony-stimulating factor.

MDD major depressive disorder.

MMP matrix metalloproteinase.

mRNA messenger RNA.

MS-222 Tricaine methanesulfonate.

MTC maximum tolerated concentration.

NA noradrenaline.

NF-κB nuclear factor kappa B.

NICE The National Institute for Health and Care Excellence.

NK natural killer cell.

NLR neutrophil to lymphocyte ratio.

NO· nitric oxide.

NOEC No Observed Effect Concentration.

NSAID nonsteroidal anti-inflammatory drug.

PECAM-1 platelet endothelial cell adhesion molecule-1.

PGE2 prostaglandin E2.

PKA protein kinase A.

PM2.5 particulate matter with a diameter of 2.5 micrometers or less.

RO reverse osmosis.

ROS reactive oxygen species.

Rp-cAMPs ribose-modified cyclic adenosine monophosphates.

SARI serotonin antagonist and reuptake inhibitor.

SerpinA3 α-1-antichymotrypsin.

SNRI serotonin-norepinephrine reuptake inhibitor.

SSRI selective serotonin reuptake inhibitor.

TCA tricyclic antidepressant.

TCR T-cell receptor.

TGF-β transforming growth factor beta.

Th1 Type 1 helper T cells.

Th17 T helper 17 cell.

Th2 Type 2 helper T cells.

TLR toll-like receptor.

TLR4 toll-like receptor 4.

TNBS trinitrobenzene sulfonic acid.

TNF tumor necrosis factor.

TNFR2 Tumor necrosis factor receptor type II.

TRAF6 tumor necrosis factor receptor-associated factor 6.

Treg natural regulatory T cells.

TRZ trazodone.

TSPO 18 kDa translocator protein.

TSST toxic shock syndrome toxin.

VCAM-1 vascular cell adhesion protein 1.

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Chapter 1

The relationship between inflammation, brain physiology and behaviour

1.1 Abstract

The relationship between the brain, immune system, and inflammation is a complex and vital aspect of maintaining brain health throughout one's lifespan. There is mounting evidence that links peripheral immune responses to various neuropsychiatric disorders, including depression. However, depression remains a significant cause of disability worldwide, and current antidepressant medications have limited efficacy in achieving full remission for many patients.

Recently, there has been increased interest in the use of non-steroidal anti-inflammatory drugs (NSAIDs) as a co-treatment for depression, and there is a need for the development of biomarkers and treatments that target the inflammatory response. Understanding the causal link between inflammatory responses and disease outcomes is crucial, particularly given the public health implications of inflammation induced by lifestyle. An interdisciplinary approach is required to quantify this link and develop effective treatments for mental health disorders associated with chronic inflammation. Depression and inflammation involve a complex interplay of immune responses and neurotransmitter metabolism, making it a challenging biological phenomenon to com-

prehend fully.

To better understand the underlying mechanisms, an evidence map has been created to visualise the existing literature, identify gaps in knowledge, and highlight areas that require further research. By gaining a better understanding of the components involved in inflammation and depression, more effective treatments can be developed, improving outcomes for those affected by these conditions.

1.2 The role of inflammation in depressive disorders

1.3 Inflammatory burden in the modern world

The immune system and inflammatory processes play a central role in a wide variety of human diseases. Chronic inflammation represents an important contributing factor in many diseases that make up a large percentage of the leading causes of death in the world, such as cardiovascular disease, obesity, multiple sclerosis, diabetes, and cancer (Furman et al., 2017, 2019; Netea et al., 2017; Slavich, 2015; Bennett et al., 2018; World Health Organization, 2021).

Acute inflammatory responses are characterized by the temporally restricted upregulation of inflammatory activity that occurs when a threat is present, and that resolves once the threat has passed (Furman et al., 2019; Kotas and Medzhitov, 2015; Straub, 2017; Fullerton and Gilroy, 2016). Unlike acute inflammation, chronic inflammation is a type of inflammation that persists over an extended period, typically for several months or years and is characterized by the activation of immune components that are often distinct from those engaged during an acute immune response (Furman et al., 2019; Straub, 2017; Calder et al., 2013). It's a long-term response that can result from a variety of factors, including chronic infections and autoimmune disorders.

The presence of certain social, psychological, environmental and biological factors has also been linked to the promotion of a state of low-grade chronic inflammation (Furman et al., 2019). While some of these factors are non-modifiable, such as chronic infection and chronic inflammatory systemic diseases like rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, some are factors typical of modern lifestyles that are at least partly modifiable, like physical inactivity, poor diet, nighttime

blue light exposure, environmental and industrial toxicants exposure and psychological stress (Furman et al., 2019). A growing body of evidence suggests that many of these factors, such as excess body weight, high-fat diet and pollution, can trigger these systemic low-intensity inflammatory responses in our bodies, which could persist for a large part of our life. For example, the link between high-fat diet or obesity and low-grade systemic inflammation has been well-established for over two decades (Wellen, 2005; Kim et al., 2012). It started with the discovery that tumor necrosis factor (TNF)-α, a proinflammatory cytokine, is over-expressed in the adipose tissues of rodent models of obesity (Hotamisligil et al., 1993; Sethi and Hotamisligil, 1999) and adipose tissue and muscle of obese humans (Hotamisligil et al., 1995; Kern et al., 1995; Saghizadeh et al., 1996).

High-fat diets have been shown to deregulate the gut microbiota and consequently increase endotoxin levels, such as lipopolysaccharide (LPS). At the same time, a high-fat diet alters the integrity of the intestinal barrier, making it more permeable and allowing for the increase of systemic LPS levels. Elevated LPS levels have been linked with increased levels of toll-like receptor (TLR) 4, proinflammatory cytokines and nuclear factor kappa B (NF-κB) activation, which induces expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. Concurrently, high-fat diet mice have been shown to have increased levels of protein kinase B (Akt) and forkhead box O3 (FOXO3) phosphorylation levels, which are involved in NF-κB activation, which is associated with intestinal inflammation (Kim et al., 2012).

Pollution plays an equally important role in the induction of chronic inflammation. Chronic exposure to airborne particulate matter with a diameter of 2.5 micrometers or less (PM2.5) triggers an increase in oxidised phospholipids in the lungs that mediate a systemic cellular inflammatory response through TLR4/FOXO3 oxidase-dependent mechanisms. Exposure to PM2.5 promotes the transport of monocytes from bone marrow to the systemic circulation, leading to the ingress of these cells into vascularised tissue niches, like perivascular fat and visceral adipose. These monocytes then induce the generation of superoxides (Kampfrath et al., 2011).

Due to the impact of lifestyle-induced inflammation at the population level and

its public health implications, there is an urgent need to understand and quantify the causal link between inflammatory responses of different intensities and disease outcomes, using an interdisciplinary perspective.

1.4 The role of inflammation in depressive disorders

Mental health disorders are one the main causes of disability worldwide. According to the latest data from the Institute for Health Metrics and Evaluation (IHME), in 2019, there were an estimated 970 million people with a mental health disorder, of which an estimated 280 million suffered from depressive disorders (IHME and Evaluation, 2019). The incidence of depressive disorders has been steadily increasing for over two decades, as shown in 1.1.

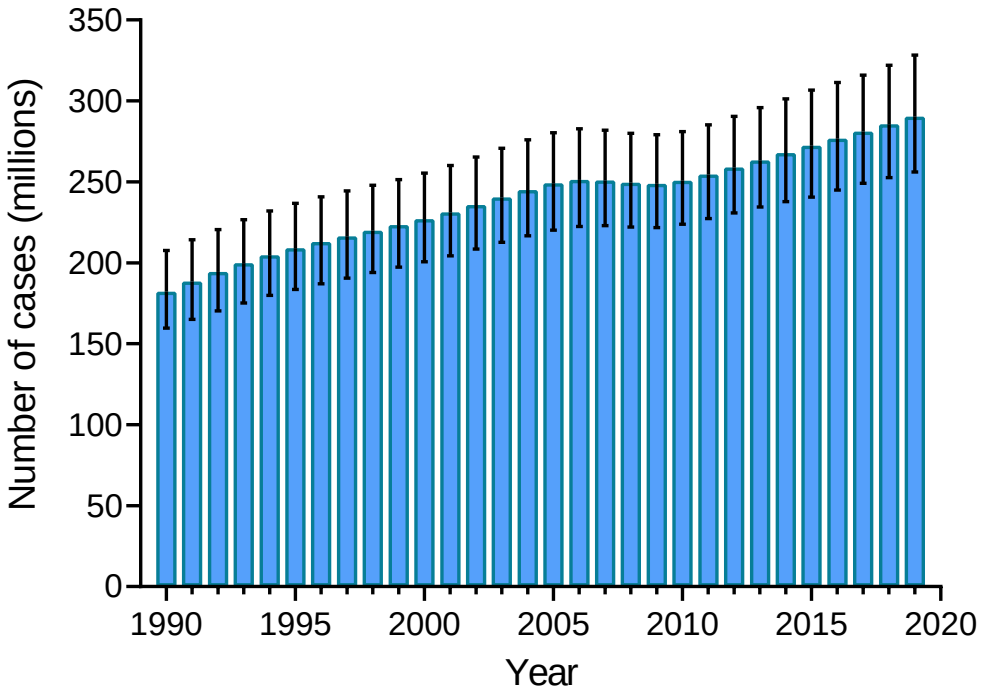


Figure 1.1 – Incidence of major depressive disorders from 1990 to 2019, expressed millions of cases. Data source: (IHME and Evaluation, 2019)

According to practice guidelines for the treatment of mood and depressive disorders across the world, from organizations such as The National Institute for Health and Care Excellence (NICE), antidepressants - alone or in combination with psychotherapy - are the recommended treatment for Major Depressive Disorder (Malhi et al., 2015;

Kennedy et al., 2016; National Institute for Health and Care Excellence (NICE), 2009; Cleare et al., 2015; Bauer et al., 2017; Munkholm et al., 2020). While guidelines were consulted to determine the recommended antidepressants by official pharmaceutical and medical bodies, a socioeconomic cost/benefit analysis specific to individual antidepressants was not undertaken, as it falls beyond the scope of this thesis. However, existing antidepressant medications can be highly effective and have been demonstrated to be more effective than the administration of a placebo (Cipriani et al., 2018), both these guidelines and several studies also acknowledge that a substantial proportion of patients do not achieve full remission. The STAR*D trial, one of the largest antidepressant studies ever conducted (National Institute of Mental Health (NIMH), 2006) reports that many patients affected by major depressive disorder do not respond to antidepressant monotherapy (Al-Harbi, 2012). Data from this trial indicate that only 36.8% of patients on antidepressants will achieve remission following initial treatment, and more than 30% will fail to achieve remission despite multiple treatment trials (Trivedi et al., 2006; Rush et al., 2006). Criticisms of the STAR*D study indicate that these numbers could be an overestimation of the number of patients achieving remission (Pigott et al., 2010; Pigott, 2015).

Research has demonstrated that a tightly controlled relationship between the brain, immune system, and signalling components of inflammation (e.g. cytokines) is critical for the maintenance of brain health throughout the life course (Kipnis, 2016). The first links between immunity and mental health disorders were made in the 1980s, revealing immunosuppression in depressed patients, specifically, blunted lymphoproliferative responses as indicated by lymphocyte transformation tests and lower numbers of T and B-cells (Maes et al., 1989, 1994, 1995; Hickie et al., 1993).

A growing number of studies have highlighted this potential link between inflammation and neuropsychiatric diseases, including major depressive disorder (Miller et al., 2009; Zalli et al., 2016). Both ill and healthy patients with major depression have been known to exhibit all of the cardinal features of inflammation, such as elevations in inflammatory cytokines and their soluble receptors in peripheral blood and cerebrospinal fluid, and elevations in peripheral blood concentrations of acute

phase proteins, chemokines, adhesion molecules, and inflammatory mediators such as prostaglandins (Zorrilla et al., 2001; Raison et al., 2006). These inflammatory markers have also been linked to individual depressive symptoms such as fatigue, cognitive dysfunction, and impaired sleep (Motivala et al., 2005; Bower et al., 2002; Meyers et al., 2005), while acute or chronic administration of cytokines has been shown to lead to the development of depressive symptoms (Birerdinc et al., 2012; Alavi et al., 2011; Musselman et al., 2001). Several longitudinal studies have also demonstrated that children who display elevated levels of inflammatory markers interleukin (IL)-6 or C-reactive protein (CRP) have an increased risk of having depression, psychosis or schizophrenia as young adults (Gimeno et al., 2009; Wium-Andersen et al., 2014; Khandaker et al., 2014; Metcalf et al., 2017; Khandaker et al., 2018).

Impaired responses to stress have been linked to several mental health disorders, such as major depressive disorder (MDD), schizophrenia and psychosis (Carol et al., 2021; Gispen-de Wied, 2000; Cooper et al., 2021; Lopez-Duran et al., 2015; Burke et al., 2005), with many affected patients presenting simultaneous activation of stress and inflammatory pathways (Cowen, 2010; Zunszain et al., 2011; Pariante and Miller, 2001; Pariante, 2017). The substantial interplay between stress and inflammatory reactions accounts for these findings. A large volume of research has established that the response to psycho-social stressors, besides activating the classic “fight or flight” response (characterised by increases in heart rate and blood pressure as well as in cortisol and catecholamines), activates key inflammatory pathways in peripheral blood mononuclear cells. These include the activation of the transcription factor NF-κB and lead to marked increases in circulating levels of proinflammatory cytokines, such as IL-6 (Miller and Raison, 2016).

Disturbances in the functionality of the hormonal system that controls stress responses, known as the hypothalamic-pituitary-adrenal (HPA), axis have also been demonstrated to be a characteristic feature of major depression (Cowen, 2010; Zunszain et al., 2011; Pariante and Miller, 2001; Pariante, 2017). Neurons in the hypothalamus secrete two hormones into the blood vessels connecting the hypothalamus and the pituitary gland: corticotropin-releasing factor (CRF) and arginine vasopressin (AVP).

These hormones stimulate the anterior pituitary gland to produce and secrete adrenocorticotropic hormone (ACTH) into the general circulation. The ACTH, in turn, induces the synthesis and release of glucocorticoids (cortisol in humans and corticosterone in mice) from the adrenal glands (Stephens and Wand, 2012). Glucocorticoids regulate peripheral functions like immunity and metabolism and represent a classic endocrine response to stress (Stephens and Wand, 2012). They are an essential component of homeostasis, and changes in their optimal levels in the blood and tissues profoundly affect the brain. Glucocorticoids have additionally been shown to regulate neuronal survival, neurogenesis, acquisition of new memories, emotional appraisal of events and immune response to stress (Herbert et al., 2006; Pariante and Miller, 2001). HPA disturbances found in patients with major depression can include increased secretion and reactivity of cortisol, elevated basal cerebrospinal fluid CRH levels, increased size as well as activity of the pituitary and adrenal glands and glucocorticoid resistance (Zunszain et al., 2011; Pariante and Miller, 2001; Pariante, 2017).

The relative contribution of glucocorticoids and inflammatory signals to major depression and the molecular and clinical mechanisms of these HPA abnormalities remain unclear (Zunszain et al., 2011; Pariante, 2017). The public health relevance of this scenario resides in the concern that lifestyle-induced low-grade chronic inflammation may hamper brain health throughout the life course. On the one side, low-grade inflammation has been linked with body ageing, cognitive decline, and age-related alteration of brain structure (Rosano et al., 2012; Gabuzda and Yankner, 2013). On the other hand, clinical research indicates that people affected by major depressive disorder often display higher levels of proinflammatory cytokines in the blood (Zalli et al., 2016), although it is still unclear whether that inflammation is the cause or the consequence of the disease.

Glucocorticoid resistance, a decrease of the responsiveness to glucocorticoids, impairs the glucocorticoid-mediated negative feedback of the HPA axis (Zunszain et al., 2011). The exact mechanism behind this resistance is unknown but likely to be related to an alteration of the glucocorticoid receptor (GR)function (Carvalho and Pariante, 2008). Evidence supporting its role in depression versus that of glucocorticoid excess

is abundant, showing that antidepressants in human, animal and cellular models tend to increase GRfunction (Pariante and Miller, 2001; Pariante, 2003; Pariante et al., 2004; Yau et al., 2007; Carvalho and Pariante, 2008; Carvalho et al., 2008; Zunszain et al., 2011).

Inflammatory responses and the HPA axis are linked by a bi-directional relationship, meaning that activation of the inflammatory response can affect neuroendocrine processes, and vice versa. An inflammatory response can stimulate HPA axis activity via a direct action of cytokines on the HPA axis, causing an elevation of systemic glucocorticoid levels (Zunszain et al., 2011; Silverman and Sternberg, 2012). Conversely, HPA axis hyperactivity (usually reflected in high levels of glucocorticoids) implies ineffective action of glucocorticoid hormones on target tissues (glucocorticoid resistance) and could lead to activation of pro-inflammatory pathways (Zunszain et al., 2011; Miller et al., 2008).

Considering that the endocrine, immune and central nervous systems communicate with each other (Zunszain et al., 2011), the question arises: can subtle changes in background inflammation levels influence our brain and behaviour?

1.5 Evidence for a link between immune and behavioural responses

Although the majority of considerations in this thesis and a large body of available research concern depression, one of the most prevalent mental health disorders, a growing number of studies have highlighted the link between peripheral immune responses and other neuropsychiatric diseases that affect behaviour (Miller et al., 2009), such as autism and schizophrenia. Furthermore, peripheral inflammatory responses have also been liked to sickness behaviour, ageing and cognitive function, which could help us further our understanding of the connection between inflammation, behaviour and brain function.

1.5.1 Depression

The analogy between sickness behaviour and depression was first drawn by Raz Yirmiya in 1999. He showed that rats treated with cytokines are less sensitive to a saccharin solution reward or the presentation of a sexually-active partner (Yirmiya et al., 1999). Some of these deficits were prevented by chronic administration of antidepressant drugs, which have little or no beneficial effects on sickness behaviour. This type of pharmacological evidence allows for the distinction of immune-induced behavioural deficits due to sickness from depressive-like behaviour. In fact, activation of the innate immune system has been shown to induce depressive-like behaviour in mice independently of sickness (Frenois et al., 2007).

The data from human studies overlaps with a large body of literature on laboratory animals. These studies demonstrate that behavioural changes overlapping with those found in depression, including anhedonia, decreased activity, cognitive dysfunction, and altered sleep, can be caused by cytokines and cytokine inducers (Dantzer et al., 2008; Miller et al., 2009).

Inflammatory markers have also been linked to individual depressive symptoms such as fatigue, cognitive dysfunction, and impaired sleep (Motivala et al., 2005; Bower et al., 2002; Meyers et al., 2005). An example can be found in the increased levels of interleukin IL-6, as well as activation of NF-κB, a primary transcription factor in the initiation of the inflammatory response in association with dysregulated sleep in depressed patients and sleep deprivation (Motivala et al., 2005; Bower et al., 2002).

There are several other lines of evidence demonstrating that both acute and chronic administration of cytokines (or cytokine inducers such as LPS or vaccination) can cause behavioural symptoms that overlap with those found in major depression, in addition to correlative data linking inflammatory markers with depressive symptoms (Miller et al., 2009). Injection of LPS to healthy volunteers resulted in acute increases in symptoms of depression and anxiety (Reichenberg et al., 2001), while administration of a *Salmonella typhi* vaccine to healthy individuals produced a depressed mood, fatigue, mental confusion, and psychomotor slowing (Brydon et al., 2008). In both cases, symptom severity correlated with increases in peripheral blood cytokine con-

centrations.

In patients with cancer or infectious disease treated with chronic administration of the proinflammatory cytokine interferon-α(interferon (IFN)-α), clinically significant depression was induced in 35-45% of persons with no psychiatric disorders previous to IFN-α treatment (Birerdinc et al., 2012; Alavi et al., 2011; Musselman et al., 2001). The depressive symptoms reported by patients undergoing this therapy present considerable overlap with idiopathic major depression: fatigue (80%), depressed mood (60%), pain (55%), gastrointestinal symptoms and tension/irritability (50%), cognitive symptoms (30%), suicidal thoughts and feelings of guilt (10%). These symptoms responded to the administration of conventional antidepressant medication (Capuron et al., 2002; Musselman et al., 2001).

Although research into the link between inflammation and depression has been focused on cytokines, it has been proposed that both acquired (e.g., T and B cell) and innate (e.g., macrophage) immune responses may be involved (Mössner et al., 2007), with basis on the discovery of increased markers of T cell activation (e.g., soluble IL-2 receptor) in depressed patients (Maes et al., 1995; Sluzewska et al., 1996). Nevertheless, while depressive symptoms are present in many patients following administration of innate immune cytokines such as IFN-αto humans (Musselman et al., 2001; Capuron et al., 2002; Alavi et al., 2011; Birerdinc et al., 2012), administration of the T cell cytokine, IL-2, has also been associated with profound changes in mental status including psychosis, delirium, and agitation (Kammula et al., 1998).

1.5.2 Schizophrenia

Several studies indicate that neuroinflammatory processes may play a role in a subgroup of schizophrenia pathogenesis (Tomasik et al., 2016). Alterations in both pro- and anti-inflammatory markers in the central nervous system have been observed. These alterations have also been found in peripheral tissues and may correlate with schizophrenia symptoms.

Approximately 40% of schizophrenia patients have been shown to display signs of immune activation, e.g. changes in IL-1B, IL-6, IL-8 and α-1-antichymotrypsin (SerpinA3) transcript levels (Fillman et al., 2013, 2014). Additionally, immunohistochemical

studies have shown that the density of microglial cells and their marker, HLA-DR, are higher in post-mortem schizophrenia brains, particularly in patients who committed suicide (Bayer et al., 1999; Radewicz et al., 2000; Steiner et al., 2006a; Fillman et al., 2013).

Signs of immune dysregulation in schizophrenia have also been observed using in vivo brain imaging. The 18 kDa translocator protein (TSPO) is expressed in the mitochondrial membrane of activated microglia. By targeting this protein in positron emission tomography studies (by measuring binding of the radiolabeled ligand, PK11195), increased binding of PK11195 has been shown in the total grey matter of recent onset patients with schizophrenia (van Berckel et al., 2008) and in the hippocampus of recovering patients (Doorduin et al., 2009). This suggests the activation of microglial cells in these regions at different stages of the disease. Additionally, an increase in the release of S100B protein, a marker of nervous system damage, into the cerebrospinal fluid (CSF) indicates signs of activation of astrocytes in schizophrenia. Increased levels of S100B have been observed in the CSF of schizophrenia patients at disease onset and in drug-naïve patients (Rothermundt et al., 2004; Steiner et al., 2006b). S100B induces the production of several other immune markers by microglia cells, including COX-2 and prostaglandin E2 (PGE2) (Najjar et al., 2013). Many other cytokines show changes in schizophrenia brains and CSF, such as IL-1 β, IL-12, and TNF-α, which can be linked to microglia activation, and IL-6, IL-10, transforming growth factor beta (TGF-β), which are secreted by activated astrocytes (Tomasik et al., 2016).

1.5.3 Maternal immune-activation model for autism

The maternal immune-activation model for autism, based on the strong association of autism diagnosis with maternal infection during gestation (Atladottir et al., 2010; Lee et al., 2015), is yet another hypothesis of immune activity affecting the brain.

By injecting pregnant mice dams with the viral analogue poly(I:C), a transient inflammatory response is triggered, which is perpetuated in the pups, changing the development and responsiveness of their immune system and rendering it more inflammatory (Choi et al., 2016; Hsiao et al., 2012; Smith et al., 2007). Subsequent to the immune response, overproduction of IL6 and high levels of Th17 cells causes abnor-

mal brain development, resulting in impaired neuronal migration and deficits in social interaction.

1.5.4 Sickness behaviour

Cytokine-induced sickness behaviour was recognised for the first time in 1995 (Aubert et al., 1995). It occurs when a peripheral infection causes a systemic immune response that, together with its accompanying storm of cytokines, affects brain function, which translates to behavioural deficits in learning, exploration and social interaction (Kelley et al., 2003; Dantzer and Kelley, 2007).

Physiological concentrations of proinflammatory cytokines that occur after infection act in the brain to induce common symptoms of sickness, such as loss of appetite, sleepiness, withdrawal from normal social activities, fever, aching joints and fatigue (Dantzer and Kelley, 2007). Clinical trials from as early as 1989 reported that the injection of recombinant cytokines into cancer patients resulted in a variety of psychological disturbances (Dantzer and Kelley, 2007, 1989; Dantzer, 2016). Systemic administration of LPS, which acts as an infection mimic, has been shown to cause broad immune activation in the meninges and choroid plexus, as well as in the brain parenchyma (Marques et al., 2009; Chen et al., 2012; Gorina et al., 2011; Pascual et al., 2012; Zhang et al., 2014). In response to inflammatory stimuli, microglia and other glia in the brain secrete their own cytokines, further proliferating the immune response (Habbas et al., 2015; Marques et al., 2009; Zhang et al., 2014).

1.5.5 Ageing and cognitive decline

Two major components for promoting autonomy and protecting against disability, dementia, and mortality as we age are brain health and cognitive functioning (Rosano et al., 2012). Evidence has shown that several markers associated with systemic levels of inflammation are also linked to risk factors for brain health decline.

Data from animal studies and disease models consistently indicate that peripheral inflammatory mediators, such as IL-6, modulate central inflammatory processes that impact cognitive function (Gadient and Otten, 1994; Vitkovic et al., 2000; Schöbitz et al., 1994). Higher IL-6 levels, for which receptors are concentrated in the hippocam-

pus and prefrontal cortex (Van Wagoner and Benveniste, 1999; Vallières et al., 2002; Gadient and Otten, 1994), are associated with smaller hippocampal and prefrontal grey matter volume and with lower executive function and memory performance in otherwise healthy middle-aged community-dwelling adults (Marsland et al., 2006, 2008).

Evidence from animal studies indicates that IL-1 affects long-term potentiation and possibly neurogenesis, and TNF-α influences cognitive function through direct effects on long-term potentiation and synaptic scaling. IL-6 is equally an important regulator of neurogenesis, with long-term exposure, as seen in normal ageing and certain neurodegenerative diseases, interfering with neurocognitive functioning by impairing adult neurogenesis (Vallières et al., 2002; Godbout and Johnson, 2004).

In patients with Alzheimer's and Parkinson's diseases, blood levels of inflammatory markers are associated with the severity of brain functional impairment (Weaver et al., 2002; Holmes et al., 2009; Yasojima et al., 2000). Additionally, in studies of patients with arthritis taking anti-inflammatory medications (nonsteroidal anti-inflammatory drugs (NSAIDs) and steroids), there has been strong evidence for decreased risk for Alzheimer's disease (McGeer et al., 1996). NSAID intake has been associated with higher cognitive function, slower progression of cognitive decline and lower risk of developing Alzheimer's disease in non-demented populations (Lleo et al., 2007; Klegeris and McGeer, 2005).

In young adults, induction of higher blood levels of proinflammatory markers via peripheral immune stimuli results in the slowing of information processing and negative mood changes within 12 hours, supported by concurrent changes in patterns of neuronal activation in functional neuroimaging data and heightened response to emotional-related stressors (Harrison et al., 2009; Brydon et al., 2008; Wright et al., 2005).

1.6 The pathophysiology of inflammation

Inflammation is commonly defined as a local response to cellular injury marked by capillary dilatation, leukocyte infiltration, redness, heat, and pain, that serves as a mechanism for initiating the elimination of noxious agents and damaged tissue (Merriam-

Webster, 2018). Regardless of the cause, the inflammatory response likely evolved as an adaptive response for restoring homeostasis (Medzhitov, 2008) and coping with damaged or malfunctioning tissue (Matzinger, 2002), whether it is caused by infection, injury or trauma, or exposure to foreign particles, irritants or pollutants (Medzhitov, 2008). On an evolutionary level, inflammation is a highly conserved phenomenon and appears to be an important first line of defence for both invertebrates and vertebrates (Ashley et al., 2012). Both unicellular organisms and more complex multicellular organisms display many of the components associated with the inflammatory cascade, such as chemotaxis and phagocytosis (Rowley, 1996).

As a physiological response of the organism to disrupted tissue homeostasis caused by physical, chemical or biological noxious stimuli or conditions, inflammation acts through the coordinated action of many cell types and mediators. The components involved in the inflammatory response depend on the nature of the initial stimulus, although a normal acute inflammatory response involves the delivery of blood components (plasma and leukocytes) to the site of infection or injury. This acute inflammatory response is initiated by tissue-resident macrophages and mast cells, leading to the production of different types of inflammatory mediators (chemokines, cytokines, vasoactive amines, eicosanoids, and products of proteolytic cascades) (Medzhitov, 2008; Monteiro and Azevedo, 2010).

The main and most immediate effect of these mediators is local, allowing the extravasation of plasma proteins and neutrophils, which are typically restricted to the blood vessels, through the postcapillary venules, to the extravascular tissues at the site of infection (or injury). When they reach the tissue site, neutrophils become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells. Activated neutrophils release the toxic contents of their granules, which include reactive oxygen species (ROS) and reactive nitrogen species, proteinase 3, cathepsin G and elastase, to the extracellular milieu (Nathan, 2006). As these highly potent effectors do not discriminate between microbial and host targets, collateral damage to host tissues is unavoidable (Nathan, 2002). Typically, a successful response results in the reestablishment of homeostasis: the elimination of the harmful

agent, followed by a resolution of the inflammation and tissue repair phase, mediated mainly by newly recruited tissue-resident macrophages. To achieve this, lipid mediators are changed from proinflammatory (e.g., prostaglandins) to anti-inflammatory and pro-resolution mediators (lipoxins, resolvins, and protectins). Tissue leucocytes undergo apoptosis and are phagocytised by the macrophages that leave the inflamed site by lymphatic drainage. This step is essential for the resolution of inflammation, as additionally to engulfing apoptotic cells, macrophages acquire a phenotype conducive to resolution afterwards, releasing anti-inflammatory signals such as interleukin IL-10 and TGF-β (Serhan and Savill, 2005). However, if the acute inflammatory response fails to neutralise and eliminate noxious stimuli or even if the clearance of apoptotic inflammatory cells from the inflamed tissue fails, the inflammatory response persists. If this happens, the inflammatory process acquires new characteristics, with the neutrophil infiltrate being replaced with macrophages and, additionally, in the case of infection, with T cells. If the combined effect of these cells is still insufficient, a chronic inflammatory state ensues, involving the formation of granulomas and tertiary lymphoid tissues (Murphy et al., 2017; Drayton et al., 2006). In this case, the invading bodies are walled off by layers of macrophages in a final attempt to protect the host (Murphy et al., 2017). Tissue damage caused by autoimmune responses (due to the persistence of self-antigens) or undegradable foreign bodies can also trigger a chronic inflammatory response.

While acute inflammation during infection or wound healing represents an adaptive response in order to restore homeostasis, chronic inflammation is outside the adaptive reaction norm, which leads to a disruption of the homeostatic state of the organism because the stimulating trigger cannot be removed (Straub and Schradin, 2016). In general, the mechanisms of systemic chronic inflammatory states are poorly understood, and the exact biological and health implications of this physiological response remain largely obscure. However, it is clear that they do not seem to fit the classic transition pattern from acute inflammation to chronic inflammation (Medzhitov, 2008), and there is an urgent need to acquire a new mechanistic understanding of the phenomenon.

From an evolutionary perspective, humans and animals have developed an array of immunological and behavioural defense mechanisms that conserve energy for fighting infection and healing wounds when needed, while still expending energy in order to remain vigilant against attack and surrrounding environment. This integrated system of stress-relevant neurocircuitry and immunity is believed to have been present during much of human evolution.

However, in nowadays modern world, there is evidence for widespread immune dysregulation. It has been suggested that this dysregularion may be the result of reduced contact with a variety of co-evolved non-lethal immunoregulatory microorganisms and parasites. These organisms, through the induction of IL-10 and TGF-β and promotion of the development of anti-inflammatory, immune cell populations (*i.e.* 'M2' macrophages and regulatory T and B cells) can reduce inflammation and suppress effector immune cells (Miller and Raison, 2016).

Commensals and symbiotes in the gut, skin, and nasal and oral cavity were once abundant in the natural environments in which humans evolved. However, in the sanitised urban environments of more developed societies, exposure to this immunoregulatory input is lacking, especially during infancy and childhood (Miller and Raison, 2016). The infectious challenges of rural environments represented the primary sources of morbidity and mortality across most of human evolution, which translated into higher infectious mortality but a lower incidence of inflammatory conditions and autoimmune diseases. Their absence has led to an environment where the lack of infectious mortality has created an exacerbated inflammatory bias. In this context, the increased psychological challenges of the modern world instigate an evolutionary inherited immunological and behavioural physiological status that accounts for the high comorbidity between depression and autoimmune, allergic and inflammatory disorders.

1.7 The gut-brain axis

The gut-brain axis and the role of the gut microbiota represent a rapidly advancing field of research in understanding the pathophysiology and treatment of neurological and psychiatric disorders. The gut-brain axis is a bidirectional communication network link-

ing the central nervous system with the enteric nervous system (the intrinsic nervous system of the gastrointestinal tract), therefore linking emotional and cognitive centers of the brain with peripheral intestinal functions (Carabotti et al., 2015). Emerging evidence suggests this bidirectional communication between the gut and the brain is mediated by the microbiome, implicating the gut microbiota as a crucial influencer of brain health and function.

Numerous studies, both preclinical and clinical, have revealed significant alterations in the composition of the gut microbiota in various mental health conditions, including depression, anxiety, autism spectrum disorder (ASD), schizophrenia, epilepsy, and Parkinson's disease. However, establishing a cause-effect relationship between gut microbiota dysbiosis and specific diseases remains challenging. It's unclear whether alterations in the gut microbiota predispose individuals to these disorders, result from the diseases themselves, or represent a combination of both factors. Moreover, inconsistencies in the gut microbiota profiles across different disease entities further complicate interpretation (Socała et al., 2021; Góralczyk-Bińkowska et al., 2022).

It's also worth noting that the gut microbiota plays a pivotal role in the metabolism of xenobiotics, which can affect the bioavailability and therapeutic efficacy of medications used to treat psychiatric and neurological disorders. Notably, certain medications used in the management of these conditions have been shown to disrupt the gut microbiota composition, potentially confounding studies examining its role in disease pathogenesis (Socała et al., 2021).

Furthermore, the gut microbiota has emerged as a key regulator of immune cells in the gut-brain axis, and dysbiosis has been associated with several inflammation-associated diseases and risk factors, including aging, diet, and stress. Given the association between intestinal dysbiosis and inflammation, which is implicated in the onset and progression of symptoms in these diseases, understanding the role of the gut microbiota in modulating immune responses and inflammation is of paramount importance (Agirman et al., 2021).

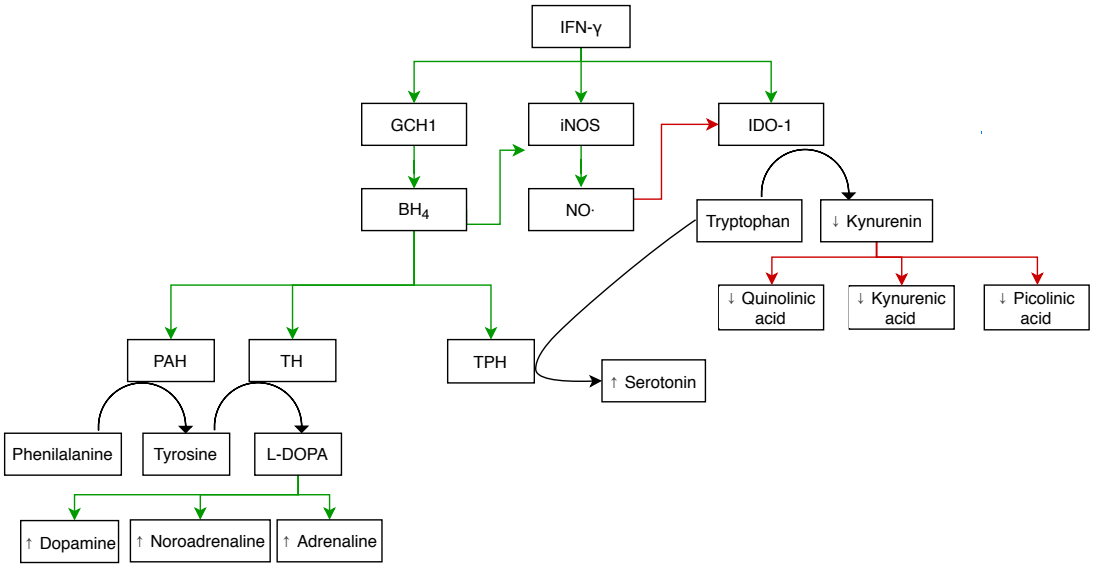
1.8 Neurotransmitter biochemistry changes associated with depressive disorders

Although the exact mechanisms that underlay the development of depressive symptoms have not yet been fully explained, it has become evident that neurotransmitter biochemistry plays a key role. The serotonergic pathway is one of the crucial neurotransmitter biosynthesis pathways, from the serotonin biosynthesis from tryptophan to its further conversion to melatonin. Equally important in the pathophysiology of depression are the dopaminergic, noradrenergic and adrenergic pathways, which start from the common intermediate levodopa (L-DOPA), whose formation is dependant on precursor molecules tyrosine and phenylalanine, and include the synthesis of dopamine, adrenaline and noradrenaline (Strasser et al., 2016).

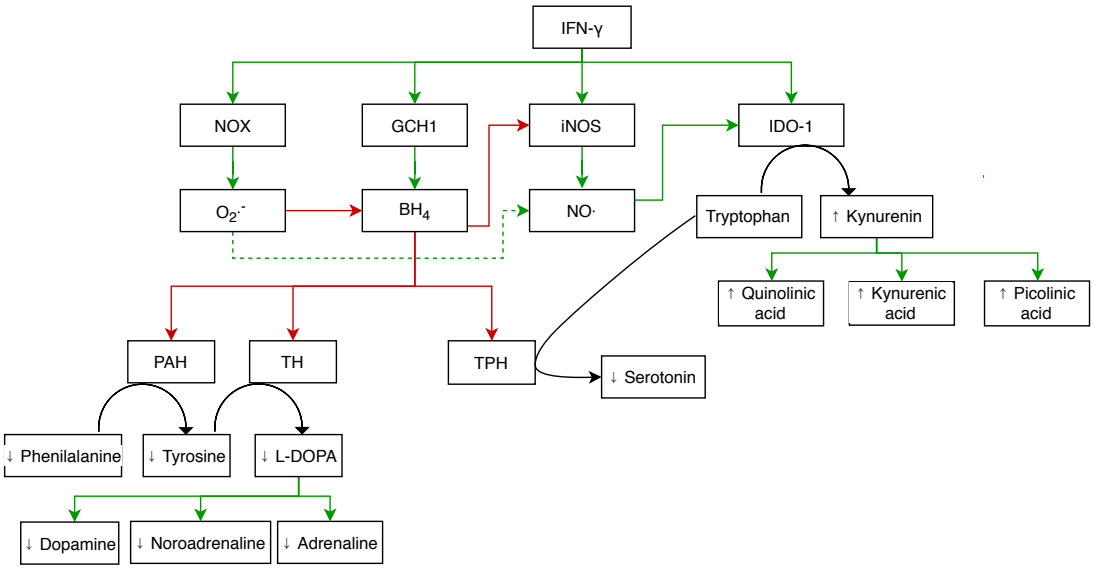
From an immunobiochemical perspective, it can be predicted that during acute immune activation, the induction of the GTP cyclohydrolase 1 (GCH-1) gene by proinflammatory cytokines like IFN-γ leads to the production of tetrahydrobiopterin (BH4). The increased BH4 availability could trigger the activity of BH4-dependent enzymes and result in increases in neurotransmitter biosynthesis (Fig. 1.2a). However, if the immune response continues, counter-regulatory pathways are initiated in parallel leading to the activation of proinflammatory cascades and the production of ROS. BH4 is sensitive to oxidation (Patel et al., 2002), which means increased concentrations of ROS that are formed during the immune response and are triggered by IFN-γ (Nathan et al., 1983) in parallel to GCH-1, deplete endogenous antioxidant pools, including BH4 concentrations (Strasser et al., 2016) (Fig. 1.2b). Disturbed function of BH4-dependent enzymes consequently diminishes the production of dopamine, noradrenaline, adrenaline and serotonin. Furthermore, While in acute immune activation, nitric oxide (NO·) suppresses the activity of indoleamine 2,3-dioxygenase (IDO)-1, leading to an increase in the formation of serotonin, in chronic immune states, inhibition of IDO-1 by NO· is no longer active, and the formation of serotonin is decreased due to the increased production of kynurenine pathway metabolites (Fig. 1.2a and 1.2b).

Current treatments for depression mainly rely on selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), alone or

in combination. The effectiveness of these treatments coupled with the link between chronic inflammation and altered dopamine, noradrenaline, adrenaline and serotonin production, highlights the importance of these pathways in the pathophysiology of depression.



(a) During acute immune activation.



(b) During chronic immune activation.

Figure 1.2 – Neurotransmitter biosynthesis during acute and chronic immune activation. (a) Cytokine Interferon-γ (IFN-γ) induces enzyme GTP-cyclohydrolase 1 (GCH1), which initiates the production of 5,6,7,8-tetrahydrobiopterin (BH4) in most cells. BH4 is a cofactor of phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH), and tryptophan hydroxylase (TPH), which are important for the biosynthesis of dopamine, noradrenaline, adrenaline, and serotonin. In parallel, several other enzymes like indoleamine 2,3-dioxygenase1 (IDO-1) and inducible nitric oxide synthase (iNOS) are activated. IDO-1 initiates the breakdown of tryptophan (Trp) via the kynurenine (Kyn) pathway. Nitric oxide (NO·) suppresses the activity of IDO-1, leading to a decrease in the levels of Quinolinic, Kynurenic and Picolinic acids. **(b)** Prolonged formation of reactive oxygen species like superoxide anion ($O_2^{\cdot -}$) by FOXO3-oxidase (NOX) wipes out 5,6,7,8-tetrahydrobiopterin (BH4). Consequently, the function of BH4-dependent enzymes is disturbed, and the production of dopamine, noradrenaline, adrenaline and serotonin is diminished. The inhibition of indoleamine 2,3-dioxygenase1 (IDO-1) by NO· is no longer active, and the formation of serotonin is decreased due to the increased production of kynurenine pathway metabolites. Green lines represent activation, red lines a decrease and black lines a breakdown reaction. L-DOPA = L-dihydroxyphenylalanine. Adapted from Strasser et al. (2016).

1.9 Evidence mapping

The pathways and processes involved in inflammation and depression are complex, with many components of immune response and neurotransmitter metabolism involved. The use of evidence mapping as a method for systematically assessing published research has become an increasingly popular approach. In order to provide a tool to facilitate the visualisation of the processes discussed so far in this chapter, an evidence map was created. This evidence map contains not only the information referenced previously in this work but also further supporting evidence for the pathways and mechanisms addressed, obtained from a further extended literature review (Fig. 1.3). All the references present in this diagram can be found in Table 1.1.

While many social, psychological, environmental and biological factors have been linked with chronic inflammatory states, obesity and high-fat diet remain one of the factors more clearly linked to altered inflammatory states in literature. As such, these two factors will be the starting point for our evidence map.

While the research presented in this section does not constitute a systematic review, it represents a simple visualisation of a comprehensive literature review that can aid in identifying gaps in knowledge and pinpointing areas where further research is required. This evidence map constitutes an important tool to facilitate the application of a systems perspective to a complex biological phenomenon through mechanistic modelling.

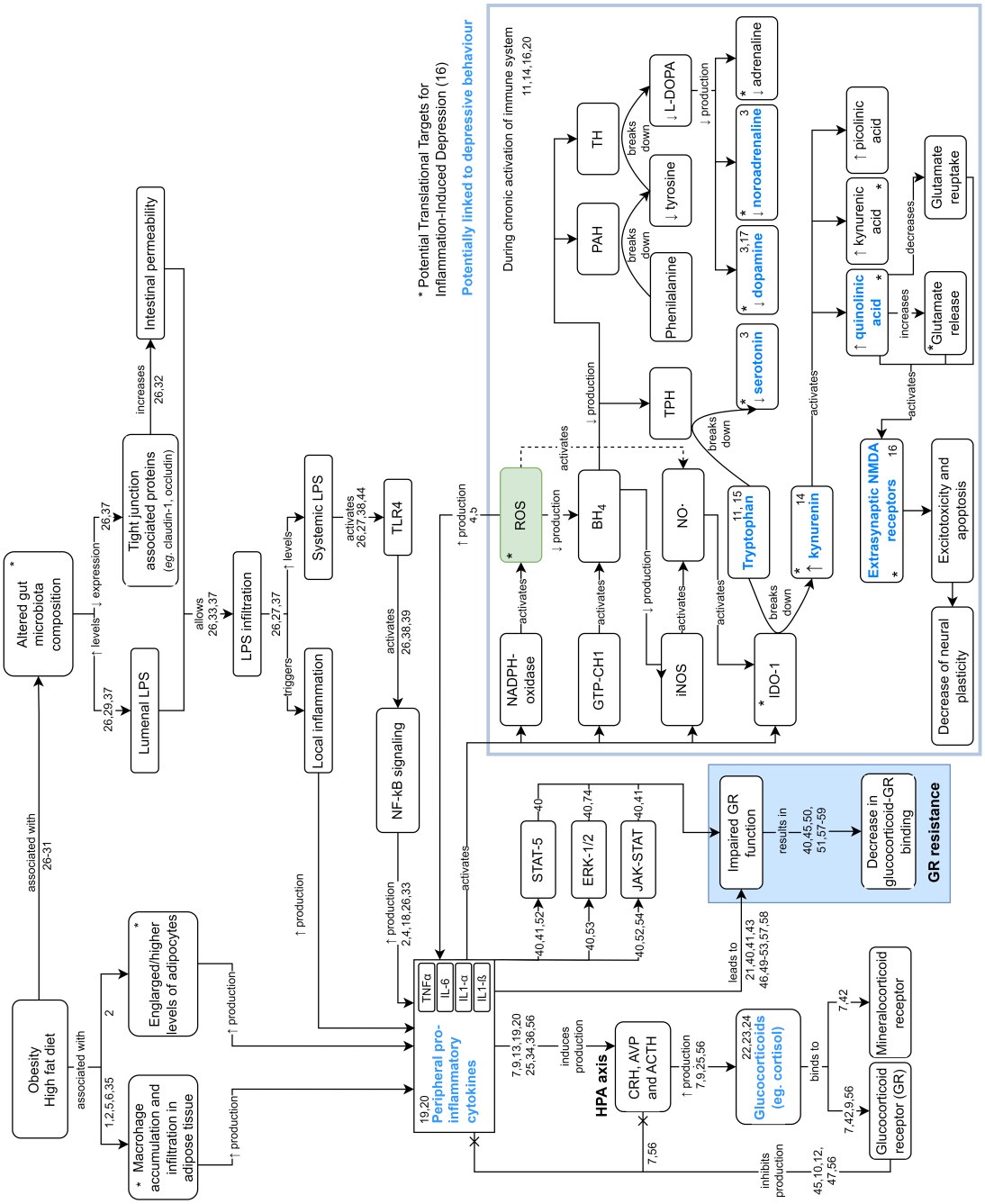


Figure 1.3 – Evidence map of inflammatory pathways in obesity and high-fat diets linked depression. High fat and obesity lead to increased levels of proinflammatory cytokines, induced by macrophage infiltration in adipose tissue, more and larger adipocytes, or increased infiltration of LPS from the gut to peripheral areas. The activation of the inflammatory response is accompanied by: induction of the HPA-axis, leading to increased production of glucocorticoids; impaired GR function; increased oxidative stress, which leads to decreased levels of dopamine, noradrenaline and adrenaline; induction of indoleamine-2,3-dioxygenase (IDO) with decreased levels of tryptophan and serotonin and consequent formation of tryptophan catabolites, which activate of extrasynaptic NMDA receptors leading to disruption of neural plasticity. BH4, Tetrahydrobioterin; ERK1/2, Extracellular signal-regulated kinase 1/2; GR, Glucocorticoid receptor; GTPCH1, Guanosine triphosphate cyclohydrolase 1; iNOS, Inducible nitric oxide synthase; JAK, Janus kinase; l-DOPA, l-3,4-dihydroxyphenylalanine; FOXO3, Nicotinamide adenine dinucleotide phosphate; NF-κB, Nuclear factor-κB; NMDA, N-methyl-D-aspartate; NO, Nitric oxide; PAH, Phenylalanine hydroxylase; ROS, Reactive oxygen species; STAT5, Signal transducer and activator of transcription 5; TH, Tyrosine hydroxylase; TLR4, Toll-like receptor 4; TPH, Tryptophan hydroxylase.

Table 1.1 – List of references cited in the evidence map in Fig. 1.3.

	Title	**Reference**
1	Inflammation, stress, and diabetes	(Wellen, 2005)
2	The role of inflammation and macrophage accumulation in the development of obesity-induced type 2 diabetes mellitus and the possible therapeutic effects of long-chain n-3 PUFA	(Oliver et al., 2010)
3	Cytokines sing the blues: Inflammation and the pathogenesis of depression.	(Raison et al., 2006)
4	Oxidative Stress in Obesity - A Critical Component in Human Diseases	(Marseglia et al., 2014)
5	Obesity is associated with macrophage accumulation in adipose tissue	(Weisberg et al., 2003)
6	Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance	(Xu et al., 2003)
7	Glucocorticoids, cytokines and brain abnormalities in depression	(Zunszain et al., 2011)
8	Serum visfatin and vaspin levels in prepubertal children - effect of obesity and weight loss after behavior modifications on their secretion and relationship with glucose metabolism	(Martos-Moreno et al., 2011)
9	Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age	(Antoni, 1993)
10	Mechanisms Generating Diversity in Glucocorticoid Receptor Signaling	(Revollo and Cidlowski, 2009)
11	Mechanisms of Inflammation-Associated Depression: Immune Influences on Tryptophan and Phenylalanine	(Strasser et al., 2016)
12	Minireview: Latest Perspectives on Antiinflammatory Actions of Glucocorticoids	(De Bosscher and Haegeman, 2009)
13	Cytokines stimulate the CRH but not the vasopressin neuronal system: evidence for a median eminence site of interleukin-6 action.	(Spinedi et al., 1992)
14	Role of the Kynurenine Metabolism Pathway in Inflammation-InducedDepression: Preclinical Approaches	(Dantzer, 2016)
15	Immune-to-Brain Communication Pathways inInflammation-Associated Sickness and Depression.	(D'Mello and Swain, 2016)
16	Inflammation and Its Discontents: TheRole of Cytokines in the Pathophysiology of Major Depression	(Miller et al., 2009)

Table 1.1 – List of references cited in the evidence map in Fig. 1.3 *(Continued)*.

	Title	Reference
17	The Role of Dopamine in Inflammation-Associated Depression: Mechanisms and Therapeutic Implications.	(Felger, 2016)
18	Molecular Control of Immune/Inflammatory Responses - Interactions Between Nuclear Factor- B and Steroid Receptor-Signaling Pathways	(McKay, 1999)
19	Relationships between interleukin-6 activity, acute phase proteins, and function of the hypothalamic-pituitary-adrenal axis in severe depression	(Maes et al., 1993)
20	The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression	(Maes et al., 2009)
21	The proinflammatory cytokine, interleukin-1alpha, reduces glucocorticoid receptor translocation and function.	(Pariante et al., 1999)
22	The diurnal variation of plasma cortisol levels in depression	(McClure, 1966)
23	Increased salivary cortisol after waking in depression.	(Bhagwagar et al., 2005)
24	Not fade away: the HPA axis and depression.	(Cowen, 2010)
25	Interleukin-1 stimulates the secretion of hypothalamic corticotro pin-releasing factor.	(Sapolsky et al., 1987)
26	High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway	(Kim et al., 2012)
27	Metabolic Endotoxemia Initiates Obesity and Insulin Resistance	(Cani et al., 2007)
28	A core gut microbiome in obese and lean twins	(Turnbaugh et al., 2009)
29	Altered Gut Microbiota and Endocannabinoid System Tone in Obese and Diabetic Leptin-Resistant Mice - Impact on Apelin Regulation in Adipose Tissue	(Geurts et al., 2011)
30	Marked alterations in the distal gut microbiome linked to diet-induced obesity	(Turnbaugh et al., 2008)
31	Human gut microbes associated with obesity	(Ley et al., 2006)
32	Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation	(de La Serre et al., 2010)
33	Immune-neuro-endocrine interactions - facts and hypotheses	(Besedovsky and Rey, 1996)

Table 1.1 – List of references cited in the evidence map in Fig. 1.3 *(Continued)*.

	Title	Reference
34	The effects of recombinant human interleukin IL-1 α, IL-1 β or IL-6 on hypothalamo-pituitary-adrenal axis activation.	(Harbuz et al., 1992)
35	Inflammation and metabolic disorders	(Hotamisligil, 2006)
36	Tumour necrosis factor-αand interleukin-2 differentially affect hippocampal serotonergic neurotransmission, behavioural activity, body temperature and hypothalamic-pituitary-adrenocortical axis activity in the rat.	(Pauli et al., 1998)
37	Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet-Induced Obesity and Diabetes in Mice	(Cani et al., 2008)
38	TLR4 links innate immunity and fatty acid-induced insulin resistance	(Shi et al., 2006)
39	Toll-like receptors as potential therapeutic targets for multiple diseases	(Zuany-Amorim et al., 2002)
40	Cytokines and Glucocorticoid Receptor Signaling	(Pace and Miller, 2009)
41	Cytokine-effects on glucocorticoid receptor function	(Pace et al., 2007)
42	Glucocorticoid receptors in major depression - relevance to pathophysiology and treatment	(Pariante and Miller, 2001)
43	Interferon-αinhibits glucocorticoid receptor-mediated gene transcription via STAT5 activation in mouse HT22 cells	(Hu et al., 2009)
44	Toll-like receptors and innate immunity	(Medzhitov, 2001)
45	Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids	(Bamberger et al., 1996)
46	Inflammatory activation is associated with a reduced glucocorticoid receptor α/ β expression ratio in monocytes of inpatients with melancholic major depressive disorder	(Carvalho et al., 2014)
47	The stress response and the hypothalamic-pituitary-adrenal axis: from molecule to melancholia	(O'Connor, 2000)
48	Mechanisms of glucocorticoid receptor signaling during inflammation	(Smoak and Cidlowski, 2004)
49	Interleukin 1 α-induced activation of p38 mitogen-activated protein kinase inhibits glucocorticoid receptor function	(Wang et al., 2004)
50	Molecular mechanisms of glucocorticoid resistance in splenocytes of socially stressed male mice	(Quan et al., 2003)

Table 1.1 – List of references cited in the evidence map in Fig. 1.3 *(Continued)*.

	Title	Reference
51	Interleukin-1 receptor type 1-deficient mice fail to develop social stress-associated glucocorticoid resistance in the spleen	(Engler et al., 2008)
52	Mitogen-activated protein kinase kinase 1/extracellular signal-regulated kinase (MEK-1/ERK) inhibitors sensitize reduced glucocorticoid response mediated by TNF-α in human epidermal keratinocytes (HaCaT)	(Onda et al., 2006)
53	Superantigen-induced corticosteroid resistance of human T cells occurs through activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK-ERK) pathway	(Li et al., 2004)
54	Glucocorticoid modulation of cytokine signaling	(Rogatsky and Ivashkiv, 2006)
55	Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase	(Rogatsky et al., 1998)
56	The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions	(McEwen et al., 1997)
57	The Physiology of Human Glucocorticoid Receptor β (hGR β) and Glucocorticoid Resistance	(Lewis-Tuffin, 2006)
58	Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant β isoform	(Webster et al., 2001)

1.10 Immunocompetence of the brain

The central nervous system has for long been regarded as an immune-privileged organ, an ”ivory tower” with tightly regulated immune access, a limited immune repertoire and an apparent separation of immune responses occurring in the brain from those in the peripheral immune system (Marin and Kipnis, 2017; Muldoon et al., 2013; Engelhardt and Sorokin, 2009; Wilson et al., 2010). Immune functions in the central nervous system (CNS) are controlled mainly by specialised tissue macrophages that reside in the parenchyma and perivascular spaces, called microglia (Nimmerjahn et al., 2005;

Raivich, 2005; Kreutzberg, 1996). These form the first line of defence for the neural parenchyma and monitor the tissue for injury or pathological changes. While most adult macrophages, resident or infiltrating, derive from classical hematopoietic stem cells, tissue-specific microglia have distinct embryonic origins, deriving from primitive myeloid progenitors/yolk-sac macrophages (Ginhoux et al., 2010; Gomez Perdiguero et al., 2014; Hanyang et al., 2017). It has been previously believed that bone marrow-derived cells (i.e. cells derived from hematopoietic stem cells) do not infiltrate the healthy CNS in adults. Instead, microglia seed the CNS early during embryonic development (Ginhoux et al., 2010). This means that blood-derived monocytes are excluded from the CNS during normal homeostasis, suggesting that the role of blood-derived monocytes is fundamentally different from that of microglia. In this scenario, the entry of antibodies, immune cells and immune mediators from the systemic circulation into the CNS is limited by the BBB. From an anatomical perspective, this barrier performs its functions thanks to tight junctions between endothelial cells and its low pinocytotic activity (Muldoon et al., 2013). However, the view that under homeostatic conditions, the traffic of leukocytes derived from hematopoietic stem cells across the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) into the CNS is prevented or relatively low (Wilson et al., 2010; Engelhardt and Coisne, 2011), has recently changed. In 2003, Ransohoff et al. suggested that the CNS should be viewed as an immunologically specialised site rather than immunologically privileged, suggesting three distinct routes of entry of leukocytes into the CNS (Ransohoff et al., 2003). Evidence also shows that activated T cells are able to breach the BBB and the BCSFB to perform immune surveillance of the CNS (Engelhardt and Coisne, 2011).

1.10.1 Mechanisms of infiltration of peripheral immune cells into the central nervous system

Multiple routes for leukocyte infiltration into the CNS have been suggested (Ransohoff et al., 2003; Alvarez and Teale, 2007; Marin and Kipnis, 2017). The CNS anatomy is unique, and understanding its structure is essential in order to dissect the possible routes of leukocyte entry. It comprises of the brain and spinal cord, which are encased not only in bone but in an additional 3 membranes that constitute the meningeal com-

partment of the brain (Ransohoff et al., 2003). These are, from the outermost to the innermost, the dural, arachnoid and pial membranes. The arterial supply of the brain parenchyma originates from terminal branches of the internal carotid arteries. When entering the parenchyma, these vessels are initially surrounded by perivascular space, which is connected to the subarachnoid space (i.e. the space between the arachnoid and pial membranes), where the vessels follow the brain surface. Additionally, arterial supply is also provided by deep penetrating branches of the internal carotid artery. In the choroid plexus, an epithelial tissue located within the ventricular system of the brain, the blood is filtrated into the cell-free low-protein CSF (Marin and Kipnis, 2017). CSF circulates from the ventricles to the subarachnoid space and is resorbed to the systemic circulation through the arachnoid villi that extend into the venous sinuses of the cerebral hemispheres (Ransohoff et al., 2003). Within the membranes surrounding the parenchymal tissue, the meningeal compartment of the CNS contains a wide variety of immune cells, making it an immunologically competent site (Bartholomäus et al., 2009; Derecki et al., 2010; Hatfield and Brown, 2015; Kim et al., 2009; Levy et al., 2007; Louveau et al., 2015; Sayed et al., 2010). The number of these immune cells can increase under inflammatory conditions, possibly on a larger scale than in the choroid plexus (Bartholomäus et al., 2009; Kim et al., 2009; Kivisäkk et al., 2003; Sayed et al., 2010; Schläger et al., 2016; Marin and Kipnis, 2017).

1.10.1.1 The CNS Castle Model

Engelhardt and Coisne (2011) proposed that the CNS possesses a unique architecture that resembles that of a medieval castle. In this model, the CSF-drained leptomeningeal/subarachnoid and perivascular spaces of the CNS represent the moat of a medieval castle, bordered on the outside by the BBB in the cerebral vasculature and the BCSFB in the choroid plexus - the outer castle wall. Having crossed the BBB or the BCSFB and reached the castle moat, the immune cells encounter a second wall, the glia limitans - the inner castle wall - composed of astrocytic foot processes and a parenchymal basement membrane. Inside the castle that is the CNS parenchyma proper, the royal family of sensitive neurons resides with their servants, the glial cells.

Within the CSF-drained castle moat, macrophages serve as guards collecting

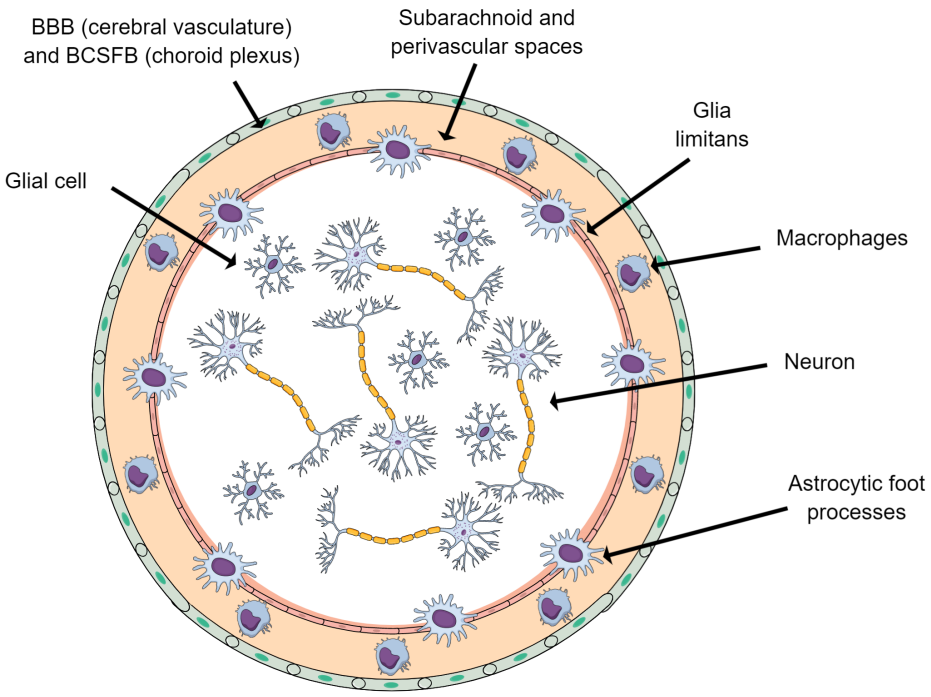


Figure 1.4 – **The Castle Moat model of the CNS.** Adapted from Muldoon et al. (2013)

all the information from within the castle, which they can present to the messengers, immune-surveying T cells. If, in their communication with the castle moat macrophages, T cells recognise their specific antigen and see that the royal family is in danger, they will become activated and open the doors in the outer wall of the castle to allow the entry of additional immune cells into the castle moat. Within the castle moat, the immune cells then mount an invasion of the castle, breaching the inner castle wall and entering the castle with the aim of eliminating any intruders. If the immune response by unknown mechanisms turns against self, that is, the castle inhabitants, chronic immune cell invasion of the castle can lead to the death of the castle inhabitants and, eventually, members of the royal family, the neurons. This leads to the neuronal deficits observed in chronic neuroinflammatory diseases of the CNS, such as multiple sclerosis. This means that inflammation and disease statuses can considerably increase leukocyte penetration into the CNS (Engelhardt and Coisne, 2011).

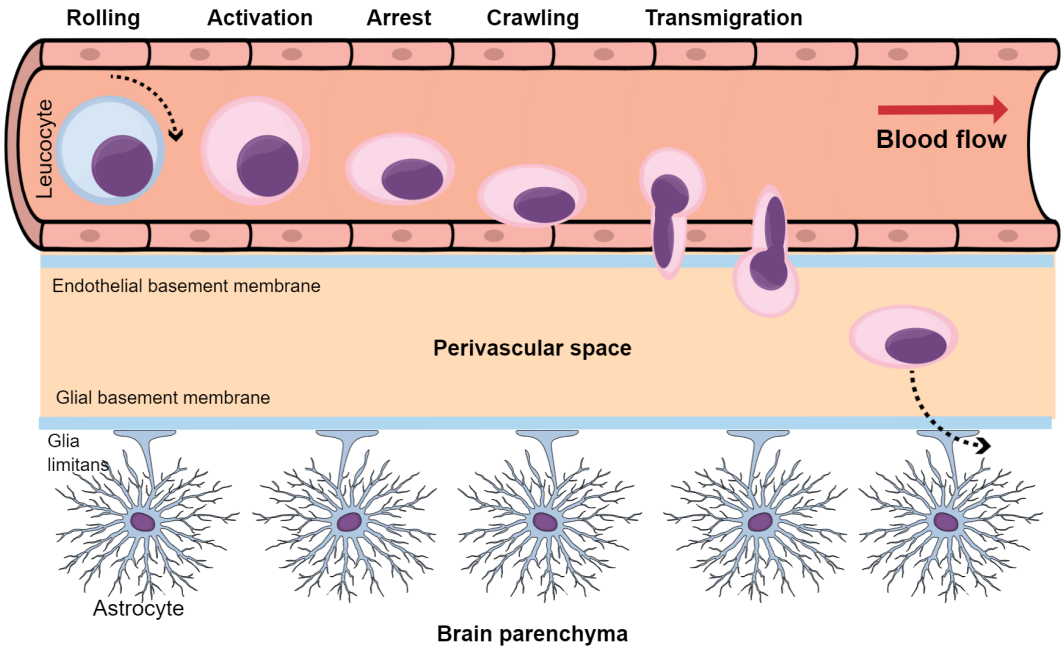


Figure 1.5 – The five steps of leukocyte penetration through the BBB: rolling, activation, arrest, crawling and transmigration. Image adapted from Takeshita and Ransohoff (2012)

1.10.1.2 Leukocyte penetration through the blood-brain barrier

Studies have shown that transendothelial leukocyte migration through the BBB is a multi-step process characterised by a series of sequential and tightly controlled steps that follow the paradigm of leukocyte extravasation across all vascular beds (Takeshita and Ransohoff, 2012; Man et al., 2007; Greenwood et al., 2011; Ley et al., 2007; Engelhardt, 2010). There are five steps in this process: rolling, activation, arrest, crawling and transmigration (Takeshita and Ransohoff, 2012) (Fig. 1.5). Brain-specific processes are determined by interacting pairs of selectins and their ligands, integrins and cell adhesion molecules (CAMs), and chemokines and chemokine receptors.

- **Rolling**: due to weak adhesion of leukocytes to endothelial cells, mainly through interactions between selectins and their carbohydrate counter-receptors (Kerfoot and Kubes, 2002), the leukocytes roll along the vascular wall with gradually reduced velocity.

- **Activation**: rolling slows leukocyte velocity allowing leukocyte activation through chemokine stimulation of G-protein-linked receptors (Engelhardt, 2008; Johnston

and Butcher, 2002; Ley, 1996). This results in functional activation of adhesion molecules along their surface (Hughes and Pfaff, 1998; Laudanna and Alon, 2006; Ward and Marelli-Berg, 2009; Rosenman et al., 1995; Man et al., 2009).

- **Arrest**: leukocytes attach to endothelial cells through interactions between integrins associated with leukocytes and CAMs on endothelial cells (Greenwood et al., 2011). The binding of these integrins to their endothelial ligands generates cytoplasmic signalling cascades in both leukocytes and endothelial cells. As a result, leukocytes arrest on endothelial cells.

- **Crawling**: After leukocytes arrest, they crawl via tightly regulated integrin/CAM interactions (Steiner et al., 2010), which initiate essential signalling within the endothelial cells and promote identification of optimal sites for transmigration (Phillipson et al., 2006).

- **Transmigration**: migration of leukocytes across CNS endothelial cells into the perivascular space (Engelhardt and Coisne, 2011). Presently, the molecular mechanisms guiding the diapedesis of leukocytes across the BBB are not well understood. One exception is the endothelial junctional molecule platelet endothelial cell adhesion molecule-1 (PECAM-1), with PECAM-1-/- mice showing increased infiltration of immune cells across the BBB (Graesser et al., 2002) demonstrating that impairment of endothelial junctions at the BBB provides a pathway for paracellular T cell diapedesis. Transmigration of leukocytes appears to be regulated by CAMs (intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1)) and chemokine signalling processes (Greenwood et al., 2011).

Following migration into the perivascular space, leukocytes must cross the glia limitans into the brain parenchyma, which is addressed below.

1.10.1.3 Leukocyte penetration through the Choroid Plexus (BCSFB)

The choroid plexus is another of the proposed routes of leukocyte penetration into the CNS (Marin and Kipnis, 2017). During inflammatory events, the number of immune

cells within the choroid plexus epithelium increases. This increase in the ordinarily low number of cells in the CSF is taken as a sign of neuroinflammation (Giunti et al., 2003; Young et al., 2011). The choroid plexus is ”activated” by inflammatory stimuli, such as peripheral infection or autoimmune diseases (Marques et al., 2009, 2007; Petito and Adkins, 2005; Reboldi et al., 2009; Shrestha et al., 2014; Young et al., 2011; Zhang et al., 2013), with ”activation” usually referring to upregulation of inflammatory cytokines (such as IL-1 β, TNF-α, and IL-6), as well as upregulation of adhesion molecules and downregulation of tight junction molecules (Marques et al., 2007, 2009; Shrestha et al., 2014; Young et al., 2011).

The expression of adhesion molecules, such as ICAM-1 and P-selectin, at baseline in the choroid plexus, allows for the creation of an environment permissive for immune-cell infiltration (Baruch et al., 2015; Kivisäkk et al., 2003). The levels of these molecules, as well as of tight junction components, are dynamically controlled by inflammatory stimuli and the cytokine milieu to regulate the amounts of cell traffic into the CSF (Baruch et al., 2015; Kunis et al., 2013; Marques et al., 2009; Zhang et al., 2013).

Other factors also limit the infiltration of immune cells through the choroid plexus. The inflammatory response to peripheral stimuli in the choroid plexus is much weaker than in other organs in the periphery (liver, spleen) and is subjected to tight temporal regulation, usually resolving within 24 h of the stimulus (Marques et al., 2009; Shrestha et al., 2014). For this reason, peripheral stimuli in the choroid plexus typically allow only for the entry of limited, nonantigen-specific immune-cell infiltrate into the CSF (Petito and Adkins, 2005; Shrestha et al., 2014; Young et al., 2011). Under autoimmune conditions, however, entry of the infiltrate is more extensive (Giunti et al., 2003; Shrestha et al., 2014).

Additionally, it has been shown that during autoimmune encephalitis, shedding of syndecans from the choroid plexus epithelium into the CSF may limit the extent of the choroid plexus infiltrate (Zhang et al., 2013). The syndecans in the CSF act like a sponge to bind released chemokines, thus sequestering them from accumulating lymphocytes.

1.10.1.4 Leukocytes in the subarachnoid and perivascular spaces

In order to proceed into the CNS parenchyma, T-cells must encounter once more their respective antigen in antigen-presenting cells (Man et al., 2007; Engelhardt and Coisne, 2011). In this process, T cells interact with many subarachnoid macrophages in search of their specific antigen, which, once recognised, would allow the cells to invade the CNS parenchyma (Bartholomäus et al., 2009). In the absence of their specific antigen, T cells remained confined to this space.

1.10.1.5 Leukocyte migration across the glia limitans

The neuropil is sealed off from the perivascular and subarachnoid spaces by the glia limitans perivascularis and glia limitans superficialis, which surround the entire surface of the brain and spinal cord, respectively (Owens et al., 2008). The glia limitans is composed of the parenchymal basement membrane and astrocytes, which embrace the entire surface of the CNS parenchyma with their foot processes.

In order for inflammatory cells to penetrate the glial limitans, the activity of matrix metalloproteinase (MMP)-2 and MMP-9 is required. MMP-2 and MMP-9 are produced by the myeloid cells surrounding the T cells in the perivascular space (Agrawal et al., 2006) and allow immune cells to breach the glia limitans and reach the CNS parenchyma (Engelhardt and Coisne, 2011).

1.10.1.6 Lymphatic vessels in the CNS

It is now widely accepted that rather than being immune-privileged, the meningeal compartment of the CNS possesses mechanisms that control the infiltration of immune cells into the parenchyma, assuring that it is under constant immune surveillance. However, the mechanism regulating the entrance and exit of these cells was poorly understood until recently. Several routes have been described regarding the mechanism of drainage of the CSF from the CNS into the periphery (Laman and Weller, 2013), such as direct drainage of cerebrospinal fluid through the cribriform plate in anatomically defined channels which connect with the nasal lymphatics (Kida et al., 1993).

The recently discovered glymphatic system represented a pathway for efficient clearance of solutes and waste from the brain (Iliff et al., 2013). It was described as

a brain-wide paravascular pathway for CSF and interstitial fluid (ISF) exchange, where the CSF enters the brain along para-arterial channels to exchange with ISF, which is, in turn, cleared from the brain along para-venous pathways.

However, the recent discovery of lymphatic vessels lining the dural sinuses by Louveau et al. (2015) represented a breakthrough in the field of neuroimmunology. The presence of these vessels gives origin to a second step in the drainage pathway of the interstitial fluid from the brain parenchyma into the periphery after it has been drained into the cerebrospinal fluid through the previously described glymphatic system (Louveau et al., 2015). All the molecular hallmarks of lymphatic endothelial cells are expressed in these newly discovered meningeal lymphatic vessels. Furthermore, they are able to carry both fluid and immune cells from the cerebrospinal fluid and are connected to the deep cervical lymph nodes (Louveau et al., 2015).

The presence of a functional and classical lymphatic system in the central nervous system represents a novel path for CSF drainage and a more conventional path for immune cells to egress the central nervous system (Louveau et al., 2015). This further suggests that the interaction between the brain and immune system may not be as limited as previously thought.

1.11 NSAIDs as a co-treatment for depression

As a result of the discovery of the relationship between inflammation and depression, selective COX-2 and non-selective COX inhibitor NSAIDs have been investigated as possible co-treatments for depression. Treatment with the NSAID acetylsalicylic acid (Aspirin) and the antidepressant drug fluoxetine led to increased remission rates in depressed patients previously nonresponsive to fluoxetine alone (Mendlewicz et al., 2006). Similarly, administration of the NSAID celecoxib in combination with the antidepressant drug reboxetine to medically healthy depressed patients resulted in greater symptomatic improvement versus patients receiving reboxetine plus placebo (Muller et al., 2006). In patients with autoimmune and inflammatory disorders, anti-inflammatory therapy was also successful in treating depressive symptoms. In a trial of etanercept, a TNF-α antagonist for the treatment of psoriasis, treatment with etaner-

cept led to significant improvement in depressive symptoms versus placebo-treated patients, independently of improvement in disease activity (Tyring et al., 2006). However, results from co-treatment trials were mixed, with some studies finding positive antidepressant effects (Pasco et al., 2010; Nery et al., 2008; Muller et al., 2006; Akhondzadeh et al., 2009; Tyring et al., 2006; Mendlewicz et al., 2006), others finding no effect (Uher et al., 2012; Fields et al., 2012; Almeida et al., 2012), and some finding detrimental effects which suggested that NSAIDs may actually reduce the antidepressant effect of SSRIs (Warner-Schmidt et al., 2011; Gallagher et al., 2012).

The variance in these results may be due to several reasons, such as differing antidepressant use and doses, the use of different NSAIDs, the study design, the age of the study population, as well as study populations with varying degrees of depressive symptomatology and presence of general medical conditions. The findings regarding the antidepressant effects of NSAID subtypes are inconsistent and negligible, and a variety of methodological improvements are required in future original treatment studies (Eyre et al., 2015). The accelerating development of biomarkers and treatments focused on the inflammatory response shows great promise of applications in psychiatry, in addition to their general clinical relevance (Miller et al., 2009). However, there is a need for further research regarding the efficacy of NSAIDs as monotherapy or as adjuncts for the treatment of depression.

1.12 Hypothesis, aims, and objectives

In this project, we will test the hypothesis that inflammation induced by exogenous inflammatory stimuli can lead to the perturbation of zebrafish behavioural phenotypes and that this effect is mediated by the modulation of neutrophils and macrophages. We also hypothesise that the key elements of the inflammation-behaviour relationship observed in mammalian pre-clinical models can be recapitulated also in the zebrafish model.

To address this hypothesis, the project will integrate experimental and computational methods. In the first phase of the project, to unravel this complex relationship, we will expose zebrafish to proinflammatory stimuli (chemicals, *i.e.* trinitrobenzene

sulfonic acid (TNBS), dextran sodium sulfate (DSS), LPS; and high-fat diet) (Oehlers et al., 2013; Progatzky et al., 2014; Kim et al., 2012) for periods ranging from 48 hours to several days. We will use *in vivo* imaging techniques to investigate the effects of the various treatments on the trafficking of two innate immune cell types, neutrophils and macrophages, which are key drivers of the inflammatory response. These data will be linked to the behaviour displayed by individual fish under various behavioural tests, quantified using automated tracking software. This approach will enable us to establish causal links between behavioural responses and specific inflammatory processes and to establish a model of inflammation-induced behaviour alteration in zebrafish. This phase of the project will allow us to develop a reproducible protocol of inflammation-induced behavioural disruption using exogenous stimuli.

The second phase of the project will be aimed at understanding the immune-modulating and anti-inflammatory properties of antidepressants by data mining large-scale transcriptomic datasets, and at validating the computational observations *in vivo* using the zebrafish inflammation model developed in the first phase of the project. The outcome of this work will contribute to addressing the hypothesis that the therapeutic effects of antidepressants may be partially due to their immunomodulatory and anti-inflammatory properties and that the differential efficacy of antidepressants can also be related to their distinct ability to modulate such pathways.

A graphical representation of the methodological steps planned for this project can be found in Fig. 1.6.

In summary, the main aim of this project is to shed light on the causal relationship between peripheral inflammation and behavioural phenotypes in the zebrafish model. To address this ambitious question, the project will have three specific objectives.

- To characterise the behavioural phenotype of zebrafish exposed to a wide set of exogenous pro-inflammatory stimuli;
- To establish a zebrafish model for the systematic assessment of the inflammation-behaviour relationship;
- To predict the immunomodulatory properties of antidepressants using transcrip-

tomic signatures;

- To validate the *in vivo* relevance of the immunomodulatory properties of antidepressants by testing their ability to interfere with inflammation-induced behaviour in the zebrafish model developed in the first phase of the project.

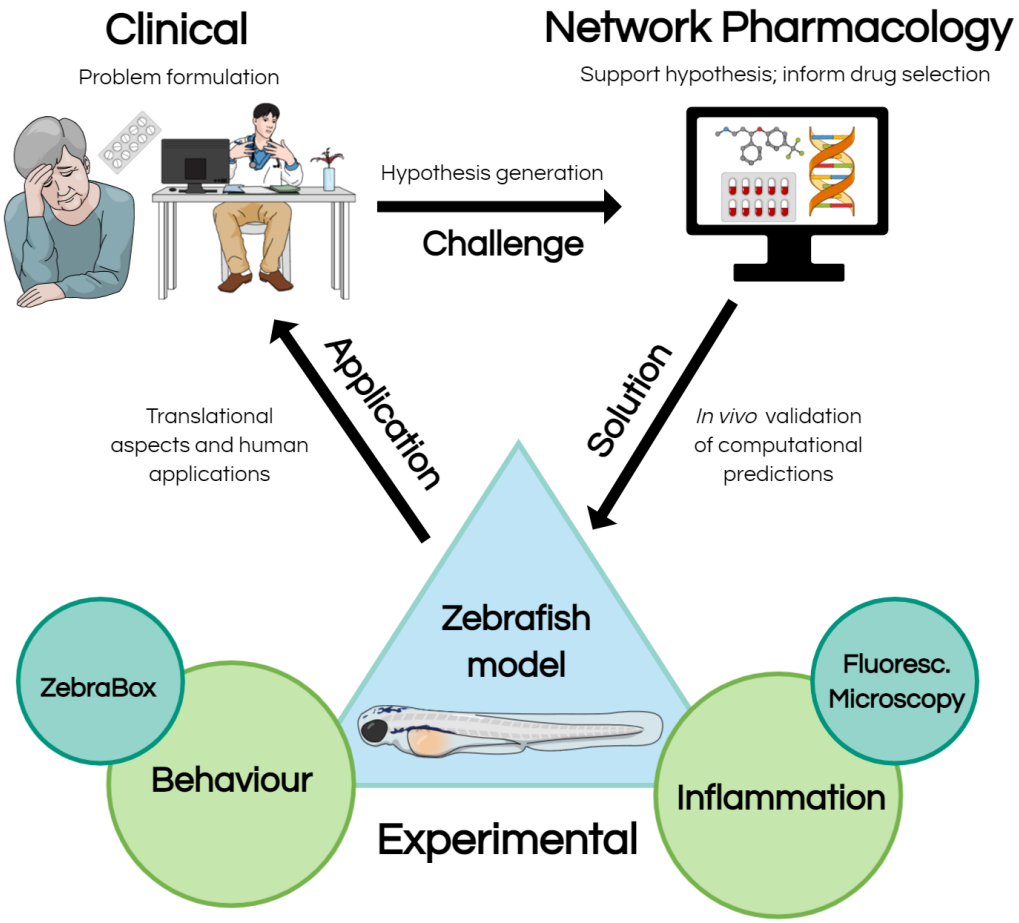


Figure 1.6 – **Methodological vision for the project.** The project starts with formulating a problem and hypothesis based on clinical data. Network pharmacology is then used to support the hypothesis and inform the selection of pharmaceutical drugs. The computational predictions are validated in a zebrafish model of inflammation-induced behaviour alterations developed concurrently with previous steps. Finally, the results can be translated into human applications.

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Chapter 2

Characterisation of behavioural responses induced by pro-inflammatory stressors in the zebrafish

2.1 Abstract

Advancing the mechanistic understanding of the link between inflammation and behaviour may lead to the identification of novel relevant antidepressant drug targets and improve the efficacy of existing treatments. While rodent models have been valuable in shedding light on the immunological and mechanistic aspects of this relationship, animal behaviour is highly variable, and many animals are needed to investigate this complex biological phenomenon, carrying high ethical and financial costs. The present work proposes that the zebrafish (*Danio rerio*) could represent a potential complementary model to investigate the relationship between inflammation and behaviour. Using a combination of immune *in vivo* imaging and behavioural phenotyping approaches, it was demonstrated that inflammation induced by the pro-inflammatory chemical dextran sulfate sodium (DSS) leads to a disruption of zebrafish behavioural phenotypes. Other pro-inflammatory treatments tested, including 2,4,6-trinitrobenzene sulfonic acid (TNBS), lipopolysaccharides (LPS), and a high-fat/high-cholesterol diet, did not show

significant effects on zebrafish behaviour. The study emphasises the importance of considering the duration and dosage of pro-inflammatory treatments and the need for further investigation to validate the inflammatory nature of behavioural alterations. Co-exposure of larvae to DSS and dexamethasone led to a recovery of the effect of DSS on inflammation, but the results obtained in behavioural tests did not suggest a direct effect of dexamethasone on behaviour. This work represents the stepping stone for future studies aimed at elucidating the exact mechanisms underlying the relationship between inflammation and behaviour in zebrafish. The novel zebrafish model described in this work has the potential to be used in more extensive drug screening activities aimed at identifying compounds able to rescue inflammation-induced behaviour before the initiation of pre-clinical work using higher vertebrate models.

2.2 Introduction

In recent years, a rapidly growing number of studies have revealed that the peripheral immune system plays a critical role in the regulation of the homeostasis of the central nervous system (CNS) in both health and disease (Herbert et al., 2006; Pariante and Miller, 2001). Since the 1970s, rodent models have led to fundamental discoveries in this area (Dantzer, 2018). For example, Kipnis et al. (2004) demonstrated that systemic immune deficiency in the mouse can lead to cognitive impairment and that the latter could be reversed by replenishment with T cells. On the other hand, several peripherally-released cytokines (interferon (IFN)-γ, interleukin (IL)-4) have been shown to regulate neuronal connectivity, social behaviour, and memory in both mice and rats (Derecki et al., 2010; Filiano et al., 2016; Bourgognon and Cavanagh, 2020). Within the wider context of neuroimmunology, the clinically observed association between peripheral inflammatory responses and behavioural disorders (Miller et al., 2009) has underpinned the need for experimental pre-clinical models able to support the characterisation of the causal relationships between the different components at play. Also, in this case, rodent models have been highly valuable in shedding light on the immunological and mechanistic aspects of such a relationship. A wide variety of rodent inflammation models have been developed in the last decades in the context of nu-

merous CNS-relevant diseases, including neurodegenerative diseases (Yang et al., 2020; Zhang et al., 2022), neuroinflammation (Yegla and Foster, 2019), depression, and anxiety (Remus and Dantzer, 2016; Cardinal et al., 2021). Common strategies to induce inflammation in those models include a high-fat diet (Cardinal et al., 2021), injection of lipopolysaccharide (LPS) (Bluthé et al., 1992; Yirmiya, 1996; Yegla and Foster, 2019; Sulakhiya et al., 2016; Bassi et al., 2012; O'Connor et al., 2009b; Walker et al., 2013), IL-1 (Kent et al., 1992), tumor necrosis factor (TNF) (Bluthé et al., 1991, 1994), IFN-α (Fischer et al., 2015), the viral mimetic Poly I:C (Gibney et al., 2013), dextran sodium sulfate (DSS) (Zonis et al., 2015; Chen et al., 2015), trinitrobenzene sulfonic acid (TNBS) (Zhou et al., 2022a; Bao et al., 2022), copper (II) sulfate ($CuSO_4$) (Kumar et al., 2019) and Bacillus Calmette-Guerin (BCG) (O'Connor et al., 2009a).

Despite the undisputed scientific value of rodent models in this field of research, rodent experiments are associated with high ethical and financial costs and are characterised by low throughput potential (Margiotta-Casaluci et al., 2019). Typical studies on inflammation-induced depression in mice can involve between 5 and 15 animals per treatment group, sometimes more (Cardinal et al., 2021). However, animal behaviour is highly variable, due to a combination of genetic, environmental and experimental factors (Hånell and Marklund, 2014). This implies that whereas obvious shifts of behavioural phenotypes (e.g. those observed pathological conditions) may be easily detected with the power of a typical study design mentioned above, the same study may be underpowered for the detection of non-severe, but translationally relevant, stressor-induced behavioural perturbations. In turn, addressing these issues by using more animals would exacerbate both ethical and experimental costs (Gulinello et al., 2019). In view of these considerations, there is an urgent need to develop novel experimental models able to support inflammation biology and neuroimmunology research while reducing those financial and ethical costs.

Here we propose that the zebrafish (*Danio rerio*) has the potential to fulfil that ambition, representing a valuable addition to the current portfolio of pre-clinical models for the investigation of inflammation-induced behavioural alterations. Zebrafish are cost-effective, reproduce rapidly and abundantly, and can be housed in large numbers in

small spaces (Kalueff et al., 2014). Comparative analysis of *in vivo* drug screening assays has revealed that zebrafish are considerably less expensive to screen, require fewer drug quantities for testing, and have higher throughput and shorter turnaround times than rodent models (Steenbergen et al., 2011). Zebrafish also display a number of complex behavioural patterns which are highly comparable to rodents and humans (Legradi et al., 2015). For this reason, there are several behavioural testing approaches which can help identify depression-like indicators in zebrafish in response to physiological, genetic, environmental, and psychopharmacological alterations. Zebrafish larvae display clear behaviour phenotypes that can be used to assess the effect of compounds and pharmaceuticals in anxiety-like behaviour: phototaxis and scotophobia, a preference for light and avoidance of dark areas; hyperlocomotion, where larvae present abnormally fast swimming for an extended period; and thigmotaxis, a preference for staying close to the edge and avoiding the central open areas (Kalueff et al., 2013; Legradi et al., 2015). These behaviours can be optimally monitored through the use of rapid, automated behavioural tracking software, which can be coupled with light-dark transitions (Nguyen et al., 2014), retrieving parameters like distance travelled, speed, thigmotaxis, immobility, and dark-light transition response.

In recent years, *in vivo* transgenic zebrafish embryos have also emerged as a powerful model for the study of the pro-inflammatory innate response to immune challenges due to their rapid development and near transparency. In zebrafish, the myeloid lineage develops rapidly, with functional macrophages appearing by 12–16 hours post fertilization (hpf) and neutrophils by 16 hpf (Gray et al., 2011). Structurally, biochemically and functionally, these cells demonstrate close homology to their mammalian counterparts. Imaging of inflammation in zebrafish relies on transgenic lines expressing fluorescent reporters in the cell types of interest, several of which exist. In this project, we will focus on two types of innate immune cells that play a key role in the mediation of inflammatory responses, neutrophils and macrophages. Several neutrophil transgenic lines are available, the most neutrophil-specific being Tg(mpx:gfp)i114, a transgenic zebrafish model of inflammation that expresses GFP under the neutrophil-specific myeloperoxidase promoter (Renshaw et al., 2006). Another transgenic line commonly used for

the study of inflammatory response is Tg(mpeg1:mCherryCAAX)sh378. This line is based on the zebrafish mpeg1 promoter that drives transgene expression specifically in zebrafish embryonic macrophages, allowing visualisation of macrophage behaviour *in vivo* (Ellett et al., 2011). Macrophage membranes are fluorescently labelled using the cysteine-aliphatic-aliphatic-any amino acid (CAAX) motif to cause the prenylation of mCherry (Bojarczuk et al., 2016). Live imaging of neutrophil and macrophage distribution, while laborious, is rapid, inexpensive and can be used to study temporal changes (Oehlers et al., 2013).

The present Chapter describes a programme of work aimed at establishing a model of inflammation-induced behaviour alteration in zebrafish. Specifically, we leveraged the features described above to characterise the behavioural effects of a wide set of pro-inflammatory stimuli in zebrafish larvae.

2.2.1 Methodological approach

The experimental approach for this project was based on a decision tree aimed at minimising the use of protected animals (Fig. 2.1). According to the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 published by the UK Home Office (Home Office, 2014), larval forms of fish are protected animals only once they are capable of feeding independently. For zebrafish, this transition is typically identified at 120 hpf, equivalent to 5 days post-fertilization (dpf) (Strähle et al., 2012). Multiple compounds were screened in zebrafish under 5 dpf, and one lead compound was identified to be investigated in detail using longer-term experiments.

During the first phase, a literature review was conducted in order to identify stressors previously reported to have pro-inflammatory effects in both mammals and zebrafish and to determine the range of concentrations with the potential to elicit inflammatory responses ranging from no effect to low toxicity. Investigating acute high-intensity inflammation capable of inducing mortality and acute toxicity was beyond the scope of the present project.

Once these stressors of interest were selected, maximum tolerated concentration (MTC) experiments were conducted. These acute exposures were performed on zebrafish larvae from 3 to 5 dpf, for a total of 48 hours of exposure, and included observa-

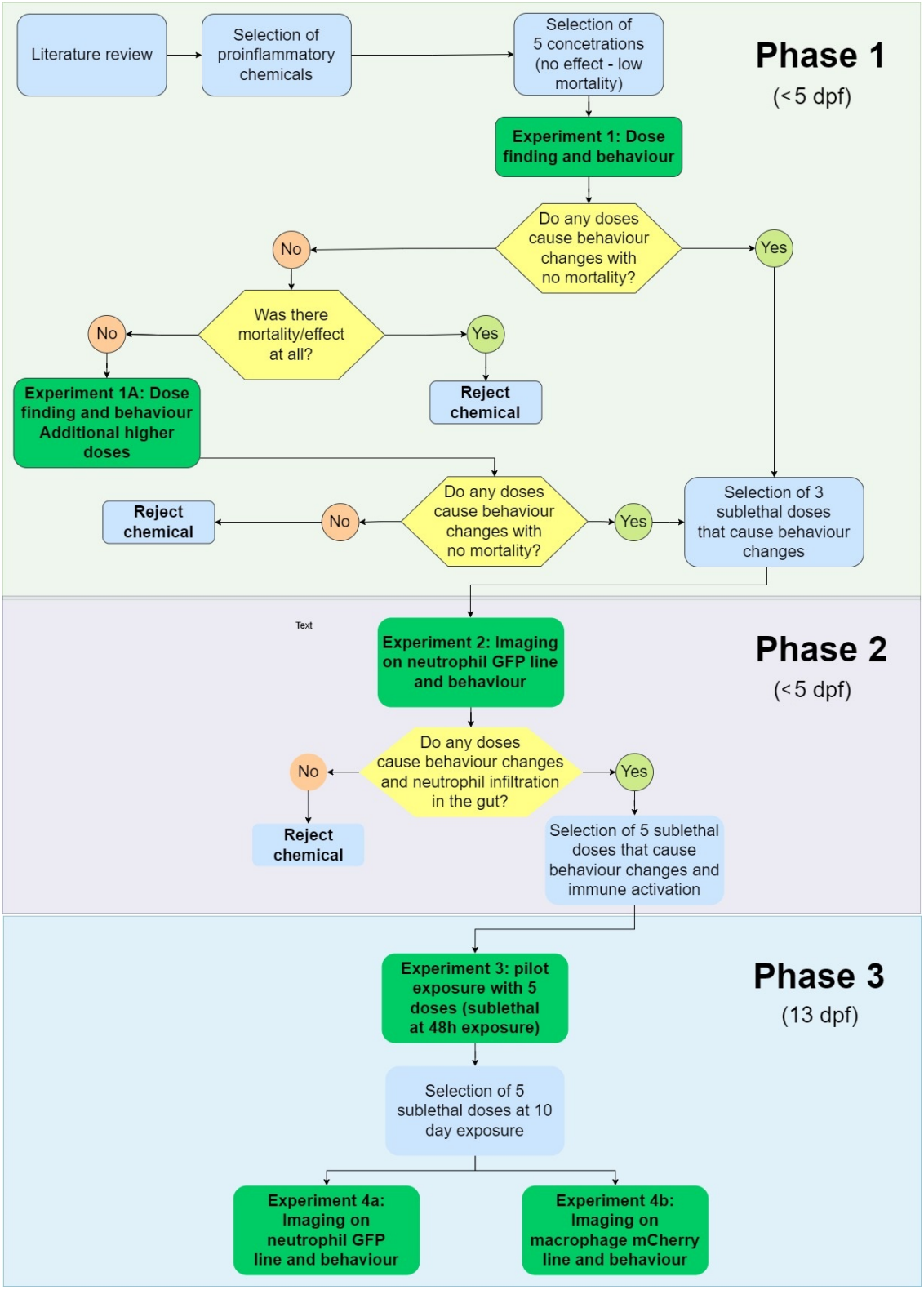


Figure 2.1 – The methodological approach for generating a zebrafish model of inflammation-induced behaviour alteration. This approach consists of three phases: a literature review and initial 48-hour screening exposures, confirmatory 48-hour exposures with transgenic lines, and 10-day exposures.

tions of mortality and developmental abnormalities, as well as behavioural tests. This step aimed to select doses leading to behavioural changes with no evident toxicological effects. When high mortality or no effect was observed using the initially set concentrations, a further experiment with lower or higher doses was conducted. At this stage, three sub-lethal doses that resulted in behavioural alterations were selected for phase two, a further 48-hour exposure using the transgenic zebrafish line Tg(mpx:GFP)i114. The exposed zebrafish underwent behavioural testing followed by *in vivo* fluorescent imaging to quantify neutrophil activation. The latter was considered as the proxy to evaluate the initiation of pro-inflammatory responses and was assessed by quantifying the infiltration of neutrophils in target areas (*e.g.* intestine, neuromast) or the systemic circulation of neutrophils away from the caudal hematopoietic tissue. Any chemicals that caused behavioural alteration and immune activation were then be selected for phase three.

Phase three focused on longer exposures from 3 to 13 dpf, for a total exposure of 10 days. Due to potential increased toxicity during more prolonged exposure, this phase started with a small pilot experiment using any sub-lethal and non-toxic concentrations from phase two. After this experiment, new sub-lethal doses were selected, and two 10-day exposure experiments were conducted using the Tg(mpx:GFP)i114 and Tg(mpeg:mCherryCAAX)Sh378/+. Each line underwent behavioural profiling followed by *in vivo* fluorescence imaging.

Once a clear inflammation-mediated behavioural phenotype was established, all phases were repeated using a co-exposure with the potent anti-inflammatory drug dexamethasone in order to attempt to rescue altered inflammation-induced behavioural phenotypes and immune effects.

2.3 Methods

2.3.1 Ethics Statement

Experiments were carried out at Brunel University London under Project License and Personnel Licences granted by the UK Home Office under the United Kingdom Animals Act (Scientific Procedures).

2.3.2 Animals

Adult AB wild-type zebrafish (*Danio rerio*) were obtained from stocks maintained at Brunel University London. Stocks were kept in a recirculating water system (ZebTec Active Blue Zebrafish Housing, Tecniplast S.p.A., Buguggiate (VA), Italy) with water temperature held at a constant 27 ± 1 ℃. Fish were cultured under a 14:10 h light:dark photoperiod and fed granular food (SPAROS, Olhao, Portugal) supplemented with brine shrimp (*Artemia salina*; ZM Fish Food, Twyford, UK) and L-strain rotifers (*Brachionus plicatilis*; ZM Fish Food, Twyford, UK), three times daily.

Tg(mpx:GFP)i114 and Tg(Mpeg MCherry CAAX)Sh378 zebrafish eggs were originally obtained from Sheffield University at the beginning of the project and raised in-house. F0 embryos received from Sheffield were kept in tall glass Petri dishes will 100 mL of E3 medium and a maximum of 50 larvae per dish. At 5 dpf, 50% of the E3 medium was replaced with system water, followed by a further 50% replacement of the medium/water mixture with fresh water every day. On day 11, larvae were added to the recirculating system. Fish were cultured at 27±1℃ in a 14:10 h light/dark photoperiod and fed granular food (SPAROS, Olhao, Portugal) and˜ live rotifers twice daily until 11 dpf. After this period, the diet was the same as for the above-described stocks. Throughout the project, a new generation of each transgenic lines was generated approximately every 14-to-18 months.

Embryos used for experiments were collected from sexually mature adults. Batches with less than 70% fertilised eggs were discarded. After microscopy imaging or behavioural analysis, larvae were terminated by schedule 1 method (Tricaine methanesulfonate (MS-222) overdose).

Sample sizes were determined according to the methodology outlined in Appendix A. The number of biological replicates utilized in each experiment is detailed in the results section of that experiment and summarized in Appendix A. It's worth noting that the variation in the number of biological replicates is influenced by the specific goals of each experiment and the availability of zebrafish larvae.

2.3.3 Test chemicals

For all exposures, a new master stock was prepared at the start of the experiment. Working stock solutions were freshly prepared each day. For each master stock, except Copper Sulphate, pH was checked and adjusted as necessary to approximately 7.5, using 1M NaOH/1M HCl. Master stocks were kept at 3 ℃ and discarded after each experiment. All chemicals were dissolved in water from a recirculating water system (ZebTEC Genotyping Solutions, Tecniplast S.p.A., Buguggiate (VA).

TNBS (CAS number 2508-19-2), LPS from *Escherichia coli* O111:B4 phenol extracted (CAS number 93572-42-0), $CuSO_4$ (CAS number 7758-98-7) and dexamethasone sodium phosphate (DSP) (CAS number 2392-39-4) were supplied by Sigma-Aldrich. DSS (CAS number 9011-18-1) was supplied by MP Biomedicals as powder.

The high-fat diet was custom ordered from SPAROS (Olhão, Portugal). Detailed composition is presented in Tables 2.1 and 2.2 .

Table 2.1 – Composition of custom ordered high-fat diet versus Zebrafeed (SPAROS, Olhão, Portugal)

	Zebrafeed	High fat diet
Crude protein, %	63	52
Crude fat, %	14	30
Fiber, %	1.8	0.1
Ash, %	12	4.2
Cholesterol, %	0	4

Table 2.2 – Ingredients of high-fat diet with added cholesterol, custom ordered from SPAROS (Olhão, Portugal)

Ingredients	%
Cod muscle powder	29.8
Fish protein hydrolizate	10
Fish gelatin	4
Wheat gluten	14
Cellulose	5
Fish oil	22
Soy lecithin	2.5
Vit & Min Premix	1.5
Vitamin E	0.2
Cholesterol	4
Monoammonium phosphate	2
Calcium silicate	5

2.3.4 Exposure of 3 dpf zebrafish larvae to pro-inflammatory chemicals for 48 hours

At 3 dpf, free-swimming hatched larvae were individually loaded into each well of a transparent 24-well plate with 1 mL of the test solution. The 24-well plates were appropriately identified with exposure group information. Due to consistently significantly different locomotor behaviour activities over the course of typical daylight workday hours, behavioural observations always started after the first one to two hours following the initiation of the light photoperiod (Kristofco et al., 2016). After loading, plates were incubated at 27±1℃ in a 14:10 h light/dark photoperiod. To ensure water quality, O_2 levels and concentration of exposure chemical in the water, the medium was replaced at 4 dpf by removing 0.5 mL of the exposure medium from each well and adding 0.5 mL of fresh test solution.

During the exposure and behaviour-tracking period, zebrafish larvae were in individual wells of 24-well plates, to avoid disturbing the larva with excessive transferring and to facilitate precise behaviour-tracking. This approach enables the linking of individual behaviour to other data collected for each specific larva, such as imaging data. The use of transparent well plates allows zebrafish larvae to visually perceive each other. This methodology aligns with the standard practice of zebrafish larvae exposure for behavioural, toxicology and drug discovery studies (OECD, 2013; Batista-Filho et al., 2021; Legradi et al., 2015; Ellis and Soanes, 2012).

All experimental observations were conducted before initiation of independent feeding at $<$120 hpf (5 dpf).

2.3.5 Exposure of 3 dpf zebrafish larvae to pro-inflammatory chemicals for 10 days

After 5 dpf, zebrafish larvae are capable of feeding independently. To preserve water quality when feeding larvae during exposures up to 13 dpf, the protocol described in section 2.3.4 was adapted. A maximum of 10 dpf larvae were loaded into each well of a 6-well plate with 10 mL of the test solution. After loading, plates were incubated at 27±1℃ in a 14:10 h light/dark photoperiod. To ensure water quality, O_2 levels and

concentration of exposure chemical in the water, the medium was refreshed daily by removing 5 mL of the exposure medium from each well and adding 5 mL of fresh test solution. At 5 dpf, larvae feeding was initiated twice a day with granular food $<$100 µm (SPAROS, Olhao, Portugal). Food remaining at the bottom of the well was aspirated daily with a pipette to preserve water quality. Larvae were transferred to a new plate when necessary due to feed getting stuck to the bottom of the wells. The last feed was at 12 dpf, allowing 24 hours of fasting before imaging. An hour after feeding, larvae were individually loaded into each well of a 24-well plate with 1 mL of the test solution.

2.3.6 Behaviour tracking - Locomotor test

Locomotor tests were performed for each exposure condition either before 5 or 13 dpf. Larval swimming patterns were observed, recorded and analysed using automated tracking software (ZebraLab, ViewPoint, Lyon, France) and associated platform (Zebrabox, ViewPoint, Lyon, France). Temperature was maintained at 27±1℃ throughout the observation period. The system was set in tracking mode, and larvae behaviour was recorded for 50 minutes. This period included a 10-minute dark acclimation period followed by a 40-minute observation period consisting of two alternating 10-minute light/dark cycles (Legradi et al., 2015). The activity was recorded and analysed across three different speed thresholds: bursting at over 5mm/s, cruising at 1 to 5 mm/s, and drifting at under 1 mm/s (Fig. 2.2). To facilitate the study of changes in thigmotactic behaviour, we defined a central and outer area of the well. Given the absence of a standardized definition for the dimensions of the centre and outer regions of wells in 24-well plates used in zebrafish experiments (Schnörr et al., 2012b; Zhou et al., 2022b; Deng et al., 2023; Golla et al., 2020), we established the central area as 40% of the total well diameter (6.2mm), based on the overall well diameter of 15.6mm. The raw endpoints acquired from the ZebraLab software and Zebrabox system include distance, duration of movement, and movement count for each of the different speed thresholds. Raw data were analysed using Fast Data Monitor software (ViewPoint, Lyon, France) and self-developed Microsoft Excel macros. Several endpoints were obtained for each larva, including the distance travelled, average speed, time spent and distance travelled in the central and outer area of the well and response to dark/light stimuli.

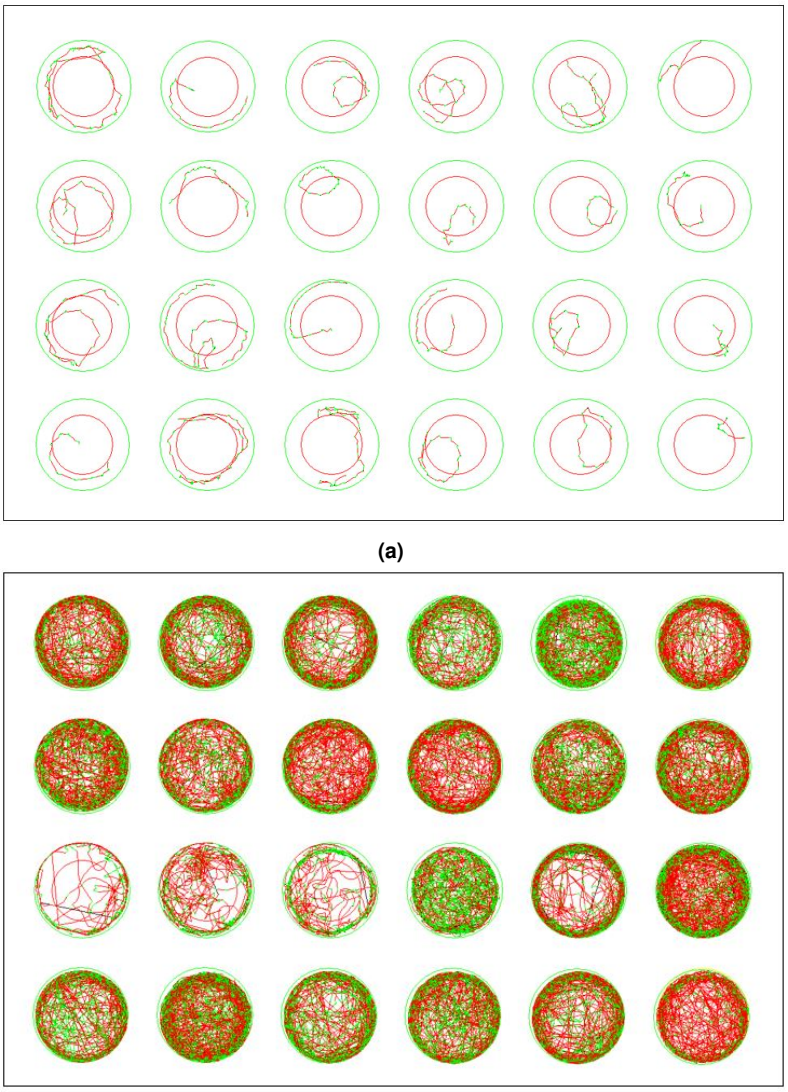


Figure 2.2 – Example output of behaviour tracking software (ZebraLab, ViewPoint, Lyon, France) and associated platform (Zebrabox, ViewPoint, Lyon France) for a 30-second (a) and 50-minute (b) period.

The sample sizes for behavioural analysis displayed minor variations across groups due to mortality and what were considered tracking glitches in some larvae analysed. These glitches occurred in a small number of wells in each experiment, despite careful signal acquisition optimisation. As seen in Fig. 2.3, excess movement was recorded in some cases in the same 30-second time period. Results for each larva were checked individually, and any well showing evidence of this glitch was removed from the analysis. The frequency of these glitches can be found in Appendix B.

While the cause of this glitch is unknown, it was hypothesised that that it happens

when larvae are immobile and reflections in the water are captured, and that this occurs with a higher frequency when there are higher numbers of empty wells or immobile larvae in a well-plate. A correlation analysis revealed a statistically significant (p=0.0049) relationship between the percentage of wells exhibiting glitches and the percentage of wells that were either empty or that contained dead larvae. A detailed breakdown of this correlation data, including additional statistical parameters, is provided in Appendix B.

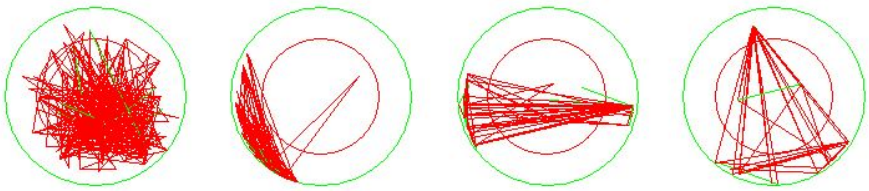


Figure 2.3 – Example of glitches in output of behaviour tracking software (ZebraLab, ViewPoint, Lyon, France) and associated platform (Zebrabox, ViewPoint, Lyon France) in a 30-second period.

2.3.7 Fluorescence imaging

After the locomotor tests, the larvae were anaesthetised individually in MS-222 (100 mg/L). After movement and response had stopped entirely, usually after 30 seconds, the larvae were transferred into an agarose solution (10 mg/mL) prepared using MS-222 at 100 mg/L as a solvent. The larvae were placed with agarose onto a microscope slide mounted with a silicone ring, quickly and carefully rotated for an unobstructed lateral view of the intestine, and depressed, so the entire intestine was in focus, using a stereo microscope. After the agarose had set, overlay images of the larvae were taken using a fluorescent Leica DMi8 microscope (Leica Microsystems, Wetzlar Germany). Unless specified, all images acquired were of the middle to the posterior intestinal area. While the 24-well plates used in behavioural tests were identified with the exposure group information, during the acquisition of microscopy images, files were deliberately labelled without exposure group information to ensure blinding.

The number of neutrophils or macrophages in the intestinal area defined in Fig. 2.4 was counted (Oehlers et al., 2013) using ImageJ software (Rasband). All images were analyzed using the same method and adhering to consistent criteria for areas and

neutrophils included, a methodology previously employed by Marmon et al. (Marmon, 2021).

Due to the time constraints of behaviour profiling, processing samples and obtaining images on the same day, sample sizes for fluorescent imaging are smaller than for behaviour. As this method is labour-intensive and depends on the user's proficiency, the quantity and quality of the images obtained improved with time, making it possible to obtain higher sample sizes in one experiment. For this reason, sample sizes are indicated for each experiment.

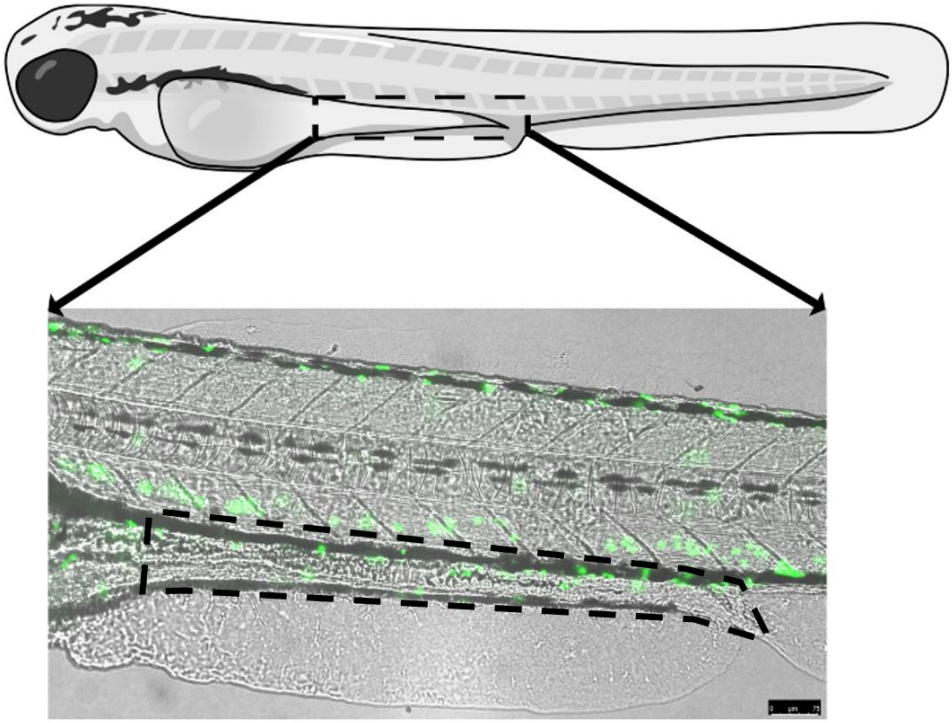


Figure 2.4 – Schematic outlining the area of the intestine in 5 dpf Tg(mpx:GFP)i114 zebrafish larvae analysed for enumeration of intestinal neutrophils and macrophages. Images have been captured using low-level background lighting with a GFP filter on a Leica DMi8 microscope (Leica Microsystems, Wetzlar Germany)

2.3.8 Statistical approach

All statistical analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, California, United States). Normality was assessed using the tests available in GraphPad Prism, including Shapiro-Wilk, D'Agostino-Pearson and Kolmogorov-Smirnov tests. Normally distributed data were analysed using ordinary one-way

ANOVA. Data that were not normally distributed were analysed using Kruskal-Wallis tests. Groups were compared using multiple comparison tests: Dunnett test for ranges of concentrations to correct for multiple comparisons; Fisher's Least Significant Difference (LSD) test for experiments comparing multiple compounds where each comparison stands alone. Box and whisker graphs display the median, and the error bars represent the 5-95 percentile; individual dots represent outliers. Dark/light response was analized using two-way ANOVA and Dunnett test for multiple comparisons.

Statistical significance is reported using asterisks as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ **** $p<0.0001$. Detailed information about all endpoints, data extraction and statistical tests can be found in Table 2.3.

To ensure a comprehensive examination of zebrafish behaviour, outliers were intentionally retained in the analysis. The decision was grounded in the hypothesis that the zebrafish exhibit a spectrum of responses to stimuli, with the potential for both high and low responders. The inclusion of outliers aligns with the study's objective of capturing the full range of behavioural variability within the population, acknowledging the potential insights that extreme responses may offer into underlying mechanisms. It is important to note that a subset of identified outliers was attributed to glitches in the behaviour-tracking software, as mentioned in 2.3.6; consequently, these instances were systematically identified and subsequently removed from the dataset. Further details regarding the frequency and underlying causes of these glitches can be found in Appendix B.

Table 2.3 – Data analysis strategy employed during the project.

Aim	Endpoint	Parameters	Data extraction strategy (per individual larvae)	Statistical tests
Identifying alterations in response to dark and light	Mean activity (mm)	30-second time bins over a 50-minute period. Alternating 10-minute periods of dark and light.		Two-way ANOVA
Identifying disruption of individual behaviour.	Total distance travelled (m)	30-second time bins over a 50-minute period. Alternating 10-minute periods of dark and light. First 10-minute dark acclimation period excluded	Sum of all movement, converted from mm to m.	**Normality:** Shapiro-Wilk, D'Agostino-Pearson and Kolmogorov Smirnov tests. **Normally distributed data:** ordinary one-way ANOVA. **Not normally distributed data:** Kruskal-Wallis tests. **Groups comparison:** Dunnett test for ranges of concentrations to correct for multiple comparisons; Fisher's Least Significant Difference (LSD) test for experiments comparing multiple compounds where each comparison stands alone.
	Average speed (mm/s)		Sum of all movement divided by total duration.	
	Distance travelled at drifting speed (%)		Sum of distance travelled at speeds $<$1mm/s, as a percentage of the total distance travelled.	
	Distance travelled at cruising speed (%)		Sum of distance travelled at speeds 1-5mm/s, as a percentage of the total distance travelled.	
	Time spent travelling at bursting speed (%)		Sum of distance travelled at speeds $>$5mm/s, as a percentage of the total distance travelled.	
	Time spent in central area (%)		Sum of duration of movements in area 2 (center), as percentage of total duration.	
	Time spent in outer area (%)		Sum of duration of movements in area 1 minus sum of duration of movements in area 2, as percentage of total duration.	
	Distance travelled in central area (%)		Sum of all movements in area 2 (center), as percentage of total distance travelled.	
	Distance travelled in outer area (%)		Sum of all movements in area 1 minus sum of all movements in area 2, as percentage of total distance travelled.	
Identifying alteration in neutrophil infiltration in the gut	Number of neutrophils	Live imaging post exposure	Count of individual neutrophils present in the mid and posterior section of the gut.	

2.4 Results

2.4.1 Baseline experiments

To establish a reproducible method for quantifying the locomotor activity of untreated zebrafish larvae, behaviour was recorded and analysed under several conditions such as different rearing mediums and different transgenic lines. The aim was to identify the protocol and the conditions that give the highest behavioural response in control fish. These conditions were then used to run the exposure experiments in the following phases of the project.

2.4.1.1 Behavioural assessment of zebrafish larvae in water *vs* E3 medium

Some studies and protocols recommend the use of E3 embryo medium, a solution of sodium chloride, potassium chloride, calcium chloride and magnesium chloride for rearing embryonic and larval zebrafish, as it offers a more stable environment than standard fish water (Meyers, 2018; Nusslein-Volhard and Dahm, 2002). Other studies use fish water taken from the main zebrafish husbandry system, usually a solution of commercial sea salts in reverse osmosis (RO) water (Truong et al., 2011; Wilson, 2012; Westerfield, 2000).

A 5-day experiment was performed to assess potential changes in baseline behaviour for zebrafish embryos raised in fish recirculating system water or E3 medium. System water was obtained from an active Tecniplast ZebTec system that did not contain fish, equipped with automated sensors for the real-time and continuous monitoring of pH 7 (7.5±0.2), conductivity (1250±50 ms) and temperature (28±1℃). Ammonia, nitrate, nitrite, alkalinity, and hardness were monitored every two weeks. E3 medium was prepared using5.0 mM NaCl, 0.17mM KCl, 0.33mM CaCl, and 0.33mM $MgSO_4$. Zebrafish embryos were raised in separate Petri dishes containing either system water or E3 medium. For each medium, a total of 36 zebrafish larvae were used. At 5 dpf, any developmental abnormalities were recorded, and behaviour was analysed through a locomotor test, as described in the methods section.

No developmental abnormalities were observed in the larvae reared in water or E3

medium. Raising zebrafish under 5 dpf in recirculating system water *vs* in E3 medium did not affect the response of larvae to light and dark conditions (Fig. 2.5, Table 2.4), but elicited slightly higher activity in the dark ($p<0.01$) (Fig. 2.6a and 2.6b). Larval preference for the outer area of the well was not altered (Fig. 2.6c). Based on these responses and to simplify the protocol, decreasing the chances for error introduction, recirculating system water was selected for further experiments.

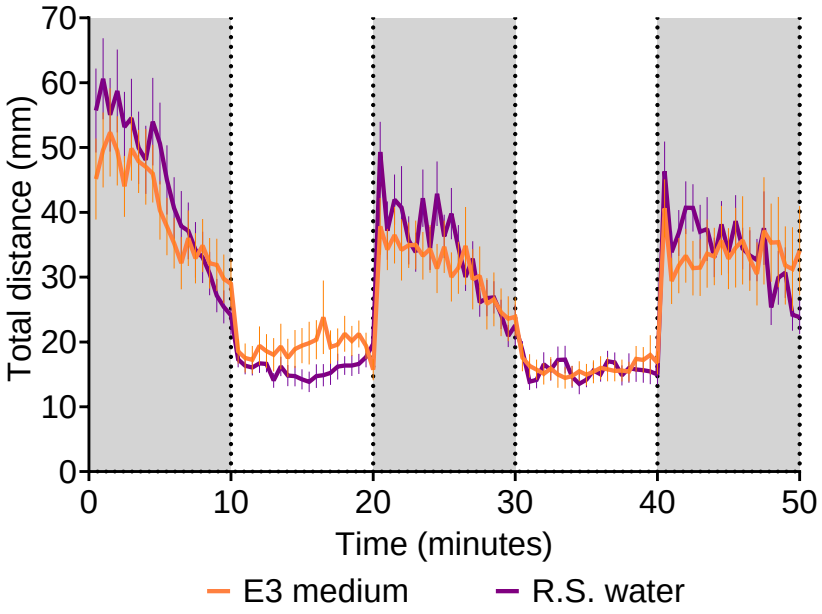
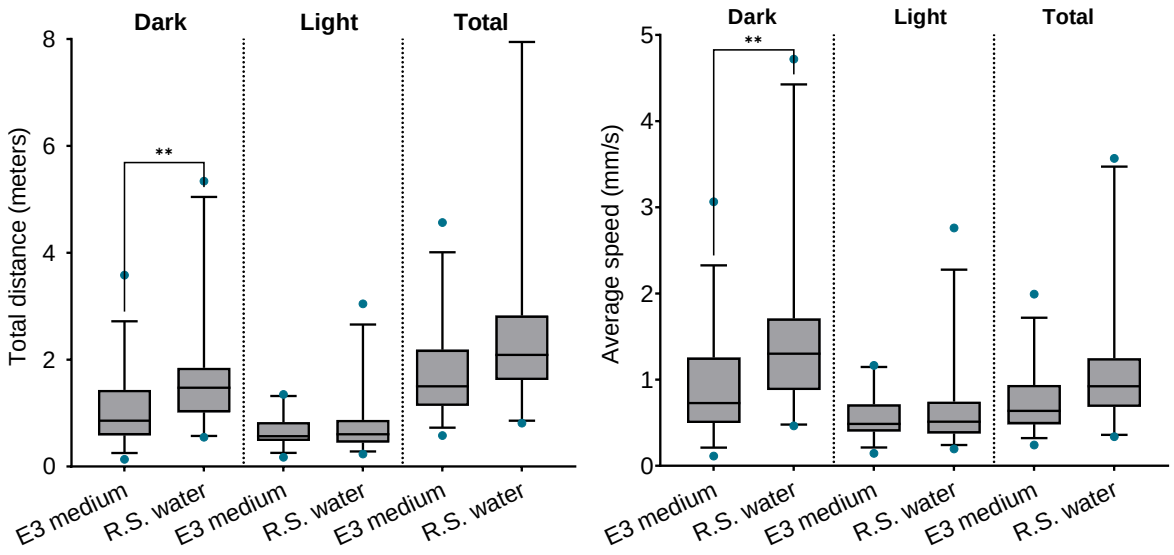


Figure 2.5 – Effect of alternating light-dark periods on locomotion in 5 dpf wild-type zebrafish larvae reared in recirculating system (R.S.) water *vs* E3 medium. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=36 larvae per group. Raising zebrafish under 5 dpf in recirculating system water *vs* in E3 medium did not affect the response of larvae to light and dark conditions.

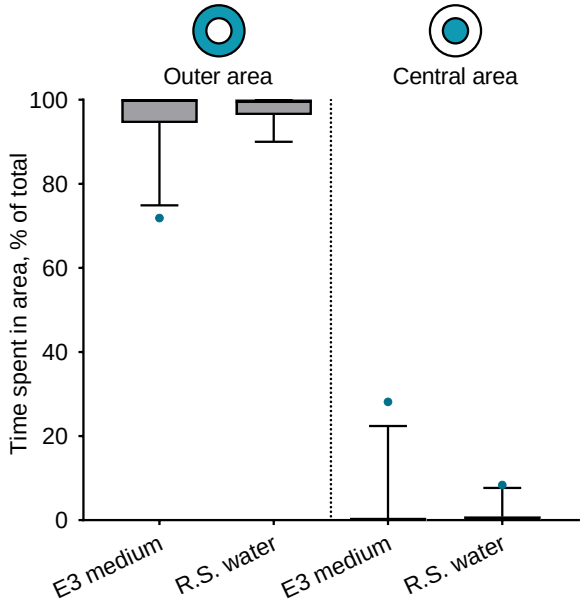
Table 2.4 – Number of time points with statistically significant difference compared to control for data in Fig. 2.5. Data represent the response to alternating light-dark periods on locomotion in in 5 dpf wild-type zebrafish larvae reared in recirculating system (R.S.) water *vs* E3 medium. The statistical methods applied are outlined in the methods section (2.3.8). N=36 larvae per group.

	R.S. water	E3 medium
Time points with statistically significant difference *vs* control	0	0



(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Time spent by larvae in the central and outer area of the well as a percent of total observed time

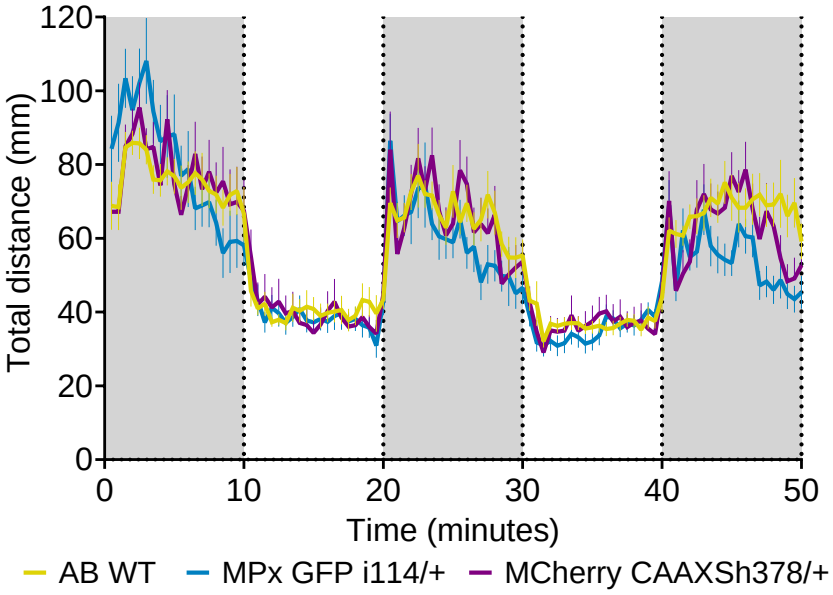
Figure 2.6 – Assessment of behavioural effects on 5dpf zebrafish larvae raised in recirculating system (R.S.) water *vs* in E3 medium. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute acclimation period has been excluded. Average distance (a), average speed (b) and time spent in the outer and central area of the well (c) were plotted. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. n=36 per group. Zebrafish larvae raised in recirculating system water are significantly more active in the dark than larvae raised in E3 medium. Thigmotactic preference is not altered by the rearing medium.

2.4.1.2 Behavioural assessment of AB wild-type zebrafish *vs* transgenic lines

The transgenic reporter lines used in the present project expressed neutrophils and macrophages tagged with fluorescent proteins (*i.e.* GFP, mCherry). No abnormal or pathological phenotype has ever been reported for these lines (Ellett et al., 2011; Renshaw et al., 2006; The Zebrafish Information Network, 2024a,b). However, no information was found in the literature concerning their behavioural profile. In order to establish a baseline behaviour profile and assess potential differences in behaviour and development between AB wild-type and transgenic lines Tg(mpx:GFP)i114 and Tg(mpeg1:mCherryCAAX)sh378, a 5-day experiment was performed. Zebrafish embryos from each line were raised in separate Petri dishes with system water. For each line, a total of 24 zebrafish larvae were used. At 5 dpf, the behaviour was analysed through a locomotor test, as described in section 2.3.6.

While in the dark/light stimuli test a few time points were significantly different between AB WT and trangenic lines, these represent a very small proportion of the total 100 time points. Other behavioural endpoints studied did not vary between these transgenic lines and AB wild-type zebrafish: larvae displayed the same levels of speed and locomotion, and thigmotactic preference was not altered (Figs. 2.7 and 2.8, Table 2.5).

No morphological changes were observed in any of the studied zebrafish lines. Based on this data, AB wild-type, Tg(mpx:GFP)i114 and Tg(mpeg1:mCherryCAAX)sh378 zebrafish larvae are therefore considered to display comparable behaviour for the remainder of this project



(a) Mean distance travelled by larvae under alternating dark and light conditions.

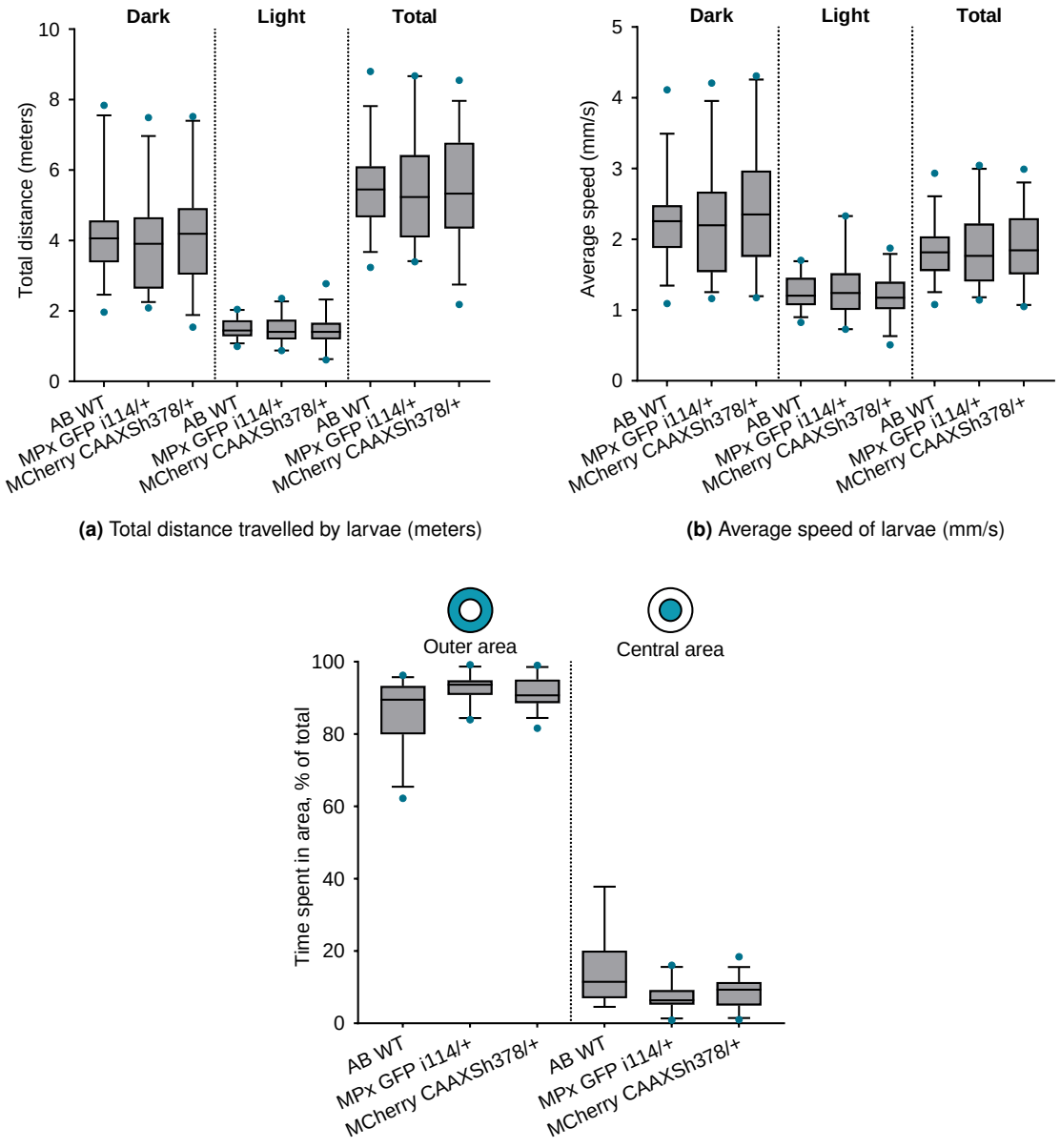
Figure 2.7 – Effect of alternating light-dark periods on locomotion in <5 dpf wild-type larval zebrafish *vs* transgenic zebrafish strains Tg(mpx:GFP)i114 and Tg(mpeg1:mCherryCAAX)sh378. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. Data are presented as mean and standard error of distance moved (mm) in 30-second intervals throughout a 50-minute session. N=32 larvae per group. Response of larvae to light and dark conditions did not vary between these transgenic lines and wild-type zebrafish.

Table 2.5 – Number of time points with statistically significant difference compared to control for data in Fig. 2.7. Data represent the response to alternating light-dark periods on locomotion in <5 dpf wild-type larval zebrafish *vs* transgenic zebrafish strains Tg(mpx:GFP)i114 and Tg(mpeg1:mCherryCAAX)sh378. The statistical methods applied are outlined in the methods section (2.3.8). N=32 larvae per group.

	AB WT*vs.* MCherry CAAXSh378/+	AB WT*vs.* MPx GFP i114/+
Time points with statistically significant difference *c*ontrol	3	13

2.4.2 Selection of pro-inflammatory chemicals

To define zebrafish mortality thresholds for different pro-inflammatory agents, preliminary 5-day MTC experiments were conducted. At 68 hours post-fertilization, after hatching, larvae were exposed to a range of concentrations of selected compounds plus a negative control. At 5 dpf, mortality, abnormal posture and unusual morphology



(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)

(c) Time spent by larvae in the central and outer area of the well as a percent of total observed time

Figure 2.8 – Assessment of behavioural differences in wild-type larval zebrafish *vs* transgenic zebrafish strains Tg(MPx GFP)i114 and Tg(Mpeg MCherry CAAX)Sh378). Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute acclimation period has been excluded. Average distance (a), average speed (b) and time spent in the outer and central area of the well (c) were plotted. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. n=36 per group. Zebrafish larvae raised in recirculating system water are significantly more active in the dark than larvae raised in E3 medium. Thigmotactic preference is not altered by the rearing medium.

were noted, and the behaviour of zebrafish larvae was analysed through a locomotor test, as described in the methods section.

2.4.2.1 TNBS

TNBS is a hapten reagent first shown to induce chronic inflammation and ulceration in the rat colon in 1989 (Morris et al., 1989). TNBS is one of the most commonly utilised chemically induced colitis animal models, and it has shown a significant consistency that reflects its extensive use during the last decades (Antoniou et al., 2016). However, the exact mechanisms that mediate TNBS pathogenesis remain unclear, and the induction method (dose, frequency of administration, animal characteristics) varies widely across studies (Silva et al., 2019; Antoniou et al., 2016).

Besides its extensive use in rodent models, TNBS has also been shown to induce inflammatory bowel disease (IBD) in zebrafish models (Oehlers et al., 2013), which can be ameliorated by co-administration of anti-inflammatory compounds (Fleming et al., 2010; Xie et al., 2021). While the behavioural effects of TNBS in zebrafish have never been investigated, previous studies have demonstrated that exposure to 25 µg/mL of TNBS induces very weak inflammation in 5 dpf zebrafish larvae, with under 5% mortality. In comparison, 50-75 µg/mL caused consistent inflammation (specifically, activation and tissue infiltration of immune cells and elevation of inflammatory markers), but with higher mortality rates (Oehlers et al., 2013). Thus the selected exposure concentrations for the MTC experiment were 6.25, 12.5, 25, 50 and 100 µg/mL.

At 68 hpf, after hatching, larvae were loaded into 24-well plates containing the selected concentrations of TNBS in recirculating system water, along with a water-only control. A total of 144 larvae were used, with 24 larvae per exposure group.

After 48h exposure to 100 µg/mL TNBS, mortality rates were 40% (Figure 2.9). No mortality was observed at the other exposure concentrations. No morphological changes were observed in any exposure group.

Although there was no apparent effect of TNBS on dark-light transition response in most exposure groups, exposure to 100 µg/mL of TNBS resulted in a more erratic pattern of activity (Fig. 2.10). However, this difference was only mild - 2 time points with statistically significant difference versus control (Table. 2.6) and could be explained by higher mortality rates and, therefore, a smaller sample size in this group. Other behavioural endpoints, such as total distance travelled, speed and thigmotaxis, were

not significantly affected (Fig. 2.11).

Previous experiments conducted with E3 medium *w*ater have also had similar results in altering the potency of TNBS. In the experiment conducted in this project, using water as a solvent, concentrations of 50 µg/mL did not affect the survival of larvae, while 100 µg/mL caused approximately 40% mortality in one experiment and no mortality in others. In other experiments conducted with water as a solvent, 100 µg/mL TNBS caused no mortality (Marmon, 2021). Conversely, for experiments with TNBS dissolved in E3 medium, 100% mortality rates were observed in larvae exposed to 50 and 100 µg/mL TNBS (Marmon, 2021).

Due to TNBS being established in the literature as a reliable method for inducing inflammation in zebrafish, TNBS was selected for additional exposure to be conducted with Tg(MPx GFP i114) zebrafish larvae to characterise both intestinal inflammation and behaviour (See Section 2.4.3.1).

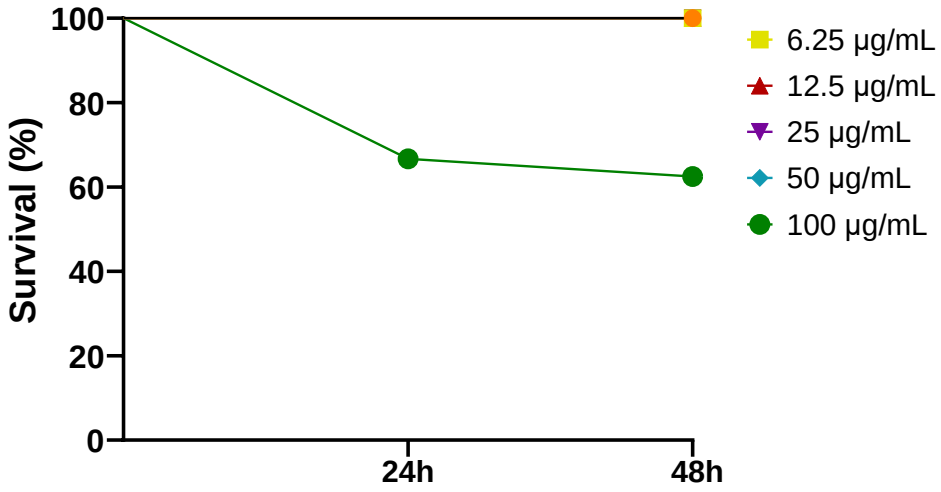
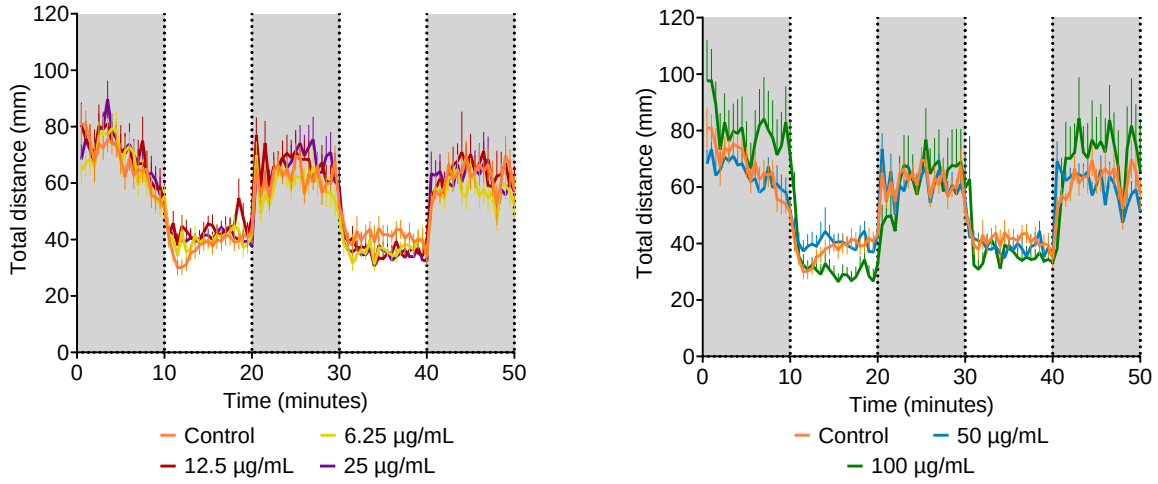


Figure 2.9 – Survival of zebrafish larvae after 48h of exposure to increasing doses of TNBS. Free swimming hatched larvae were introduced to the dilutions from 68 hpf. N=24 larvae per group. Exposure to 100 µg/mL TNBS resulted in 40% mortality. No mortality was observed in other exposure concentrations.



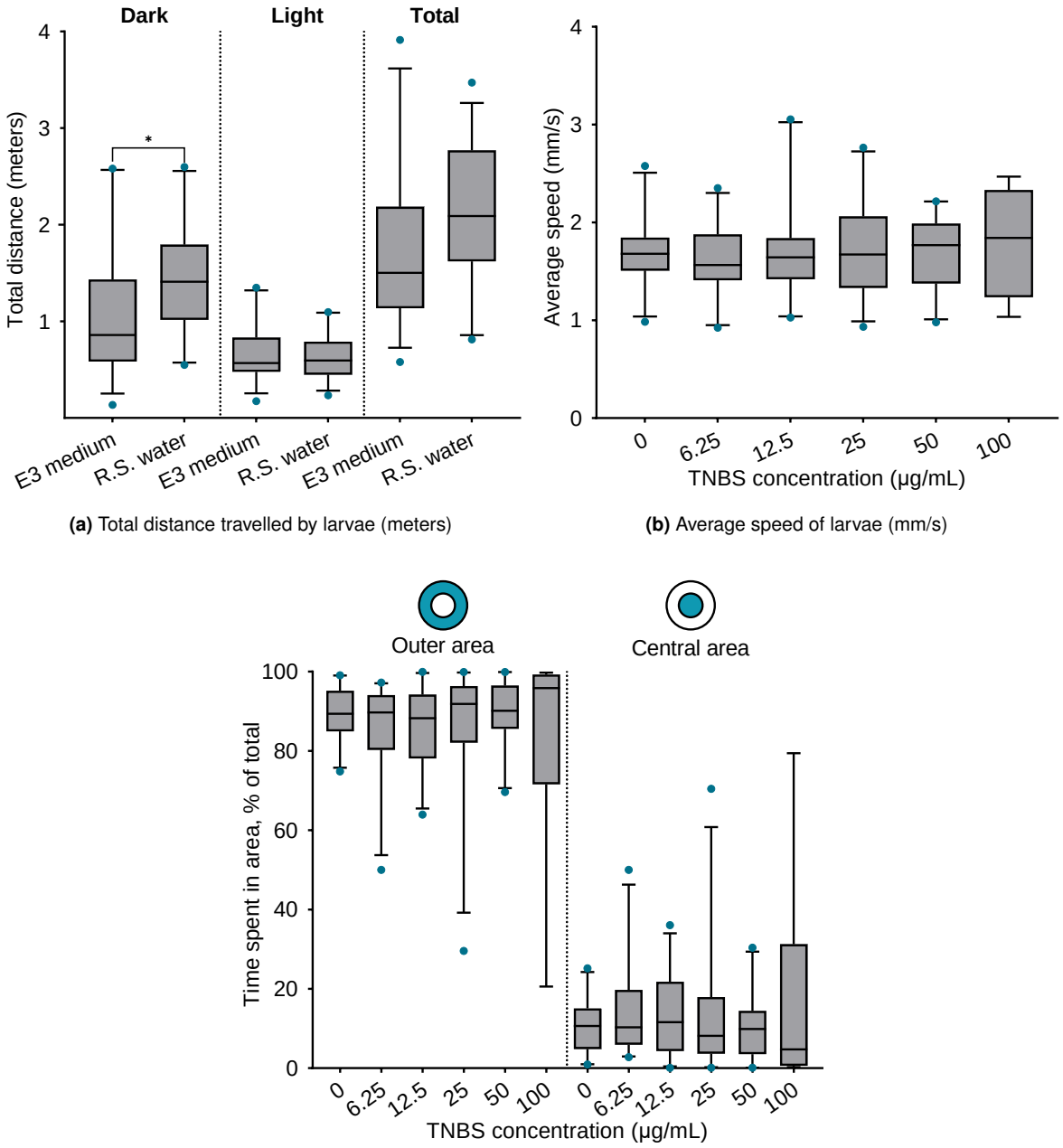
(a) Mean distance travelled by larvae under alternating dark and light conditions (6.25 to 25 µg/m)

(b) Mean distance travelled by larvae under alternating dark and light conditions (50 to 100 µg/m)

Figure 2.10 – Effect of alternating light-dark periods on locomotion in wild-type larval zebrafish after 48h of exposure to increasing doses of TNBS. Free swimming hatched larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=24 larvae per group, with the exception of 100 µg/mL, where n=12 due to increased mortality rates. Exposure to TNBS ranging from 6.25 to 100 µg/mL has no effect the response of larvae to light and dark, but causes a slightly more erratic response at 100 µg/mL. This could be explained by higher mortality rates and smaller sample size at this concentration.

Table 2.6 – Number of time points with statistically significant difference compared to control for data in Fig. 2.10. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of TNBS. The statistical methods applied are outlined in the methods section (2.3.8). N=24 larvae per group, with the exception of 100 µg/mL, where n=12 due to increased mortality rates.

	Control *vs.* 6.25 µg/mL	Control*vs.* 12.5 µg/mL	Control*vs.* 25 µg/mL	Control*vs.* 50 µg/mL	Control*vs.* 100 µg/mL
Time points with statistically significant difference *c*ontrol	0	0	0	0	2



(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)

(c) Time spent by larvae in the central and outer area of the well as a percent of total observed time

Figure 2.11 – Assessment of behavioural differences in wild-type larval zebrafish after 48h of exposure to increasing doses of TNBS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and time spent in the outer and central area of the well (c) were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=24 larvae per group, with the exception of 100 µg/mL, where n=12 due to increased mortality rates. Total distance travelled (a), average speed (b) and thigmotaxis (edge preference) (c) were not significantly affected by TNBS.

2.4.2.2 LPS

Lipopolysaccharides (LPS) have consistently been used as an inflammation model for research of anti-inflammatory drugs in zebrafish (Xie et al., 2021; Novoa et al., 2009; Yang et al., 2014; Forn-Cuní et al., 2017).

LPS, characteristic components of the cell wall of Gram-negative bacteria, stimulate toll-like receptor (TLR), which mediate pro-inflammatory signalling cascades and lead to the activation of neutrophils and macrophages and the release of inflammatory cytokines such as IL-6, IL-8, and TNF-α. This elicits multiple pathophysiological processes *in vivo*, such as metabolic changes, fever, multiple organ dysfunction syndrome, endotoxic shock, and even death (Novoa et al., 2009; Yang et al., 2014).

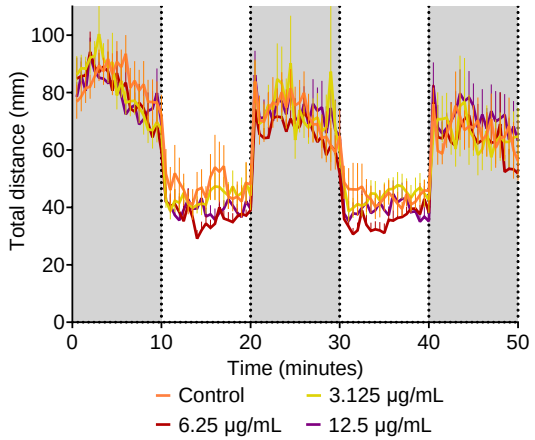
Specific strains of *Escherichia coli* (*E. coli*) are known to be zebrafish pathogens, employed to investigate inflammation, inflammatory diseases such as IBD and even gastrointestinal disease progression (Stones et al., 2017; Erika et al., 2023; Hou et al., 2016; Tan et al., 2019). LPS from *E. coli* 0111:B4 has specifically been previously used for inducing inflammation in zebrafish (Novoa et al., 2009; Yang et al., 2014; Forn-Cuní et al., 2017). While the administration is usually done by injection (Xie et al., 2021; Yang et al., 2014), there have been reports of inflammatory activity by direct exposure in water containing LPS (Novoa et al., 2009). In experiments testing LPS-induced immune activation, concentrations of 150 and 200 µg/ml of LPS from E. coli 0111:B4 induced 100% zebrafish larvae mortality on 5 dpf larvae and 60-100% mortality on 2 dpf larvae after 6h exposure. These mortality levels are likely related with a high production of proinflammatory cytokines and other molecules such as proteases, eicosanoids, and reactive oxygen and nitrogen species (Novoa et al., 2009).

It has also been reported that exposure of 4 dpf larvae LPS from *E. coli* O111:B4 for 22 hours, a concentration range of 110-120 µg/mL induces approximately 20% mortality, whereas concentrations under 100 µg/mL can induce up to 20% mortality. Age also seems to influence mortality rates, with younger larvae showing higher mortality when exposed to the same concentration of LPS*vs* older larvae (Mottaz et al., 2017). As this experiment aimed to find a range that alters behaviour without causing mortality after 48h exposure, the initial exposure concentrations selected were 3.125, 6.25,

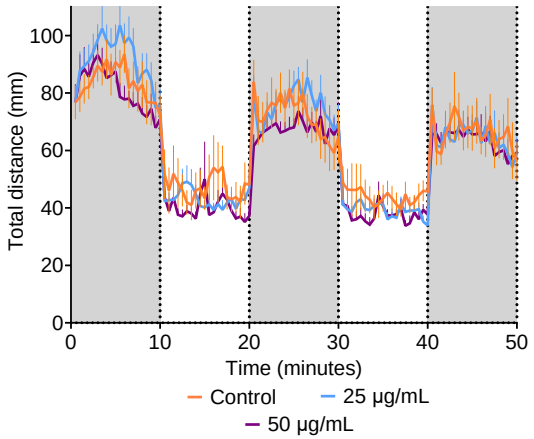
12.5, 25 and 50 µg/mL.

At 68 hpf, after hatching, larvae were loaded into 24-well plates containing the selected concentrations of LPS in recirculating system water, along with a water-only control. A total of 120 larvae were used, with 20 larvae per exposure group.

No mortality or morphological changes were observed at these concentrations. There was no notable effect of LPS on dark-light transition response in any exposure groups (Figure 2.12, Table 2.7), and other behavioural endpoints, such as total distance travelled, speed and thigmotaxis, were not significantly affected (Figure 2.13).



(a) Mean distance travelled by larvae under alternating dark and light conditions (3.125 to 12.5 µg/m)

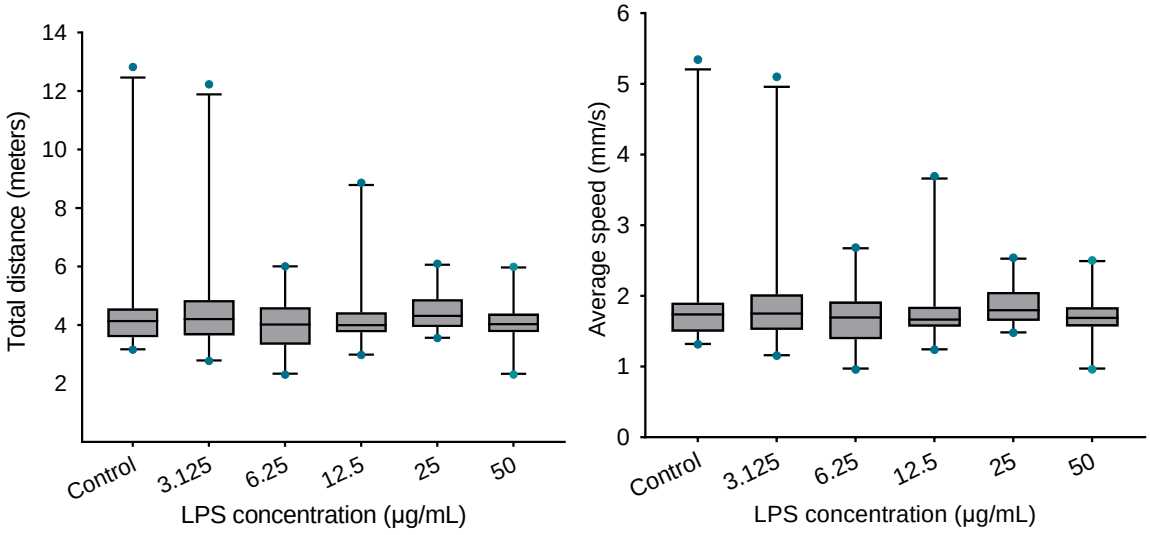


(b) Mean distance travelled by larvae under alternating dark and light conditions (25 to 50 µg/m)

Figure 2.12 – **Effect of alternating light–dark periods on locomotion in wild-type larval zebrafish after 48h of exposure to increasing doses of LPS.** Larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=20 larvae per group. Exposure to 3.125 to 50 µg/mL LPS for 48h has no effect the response of larvae to light and dark.

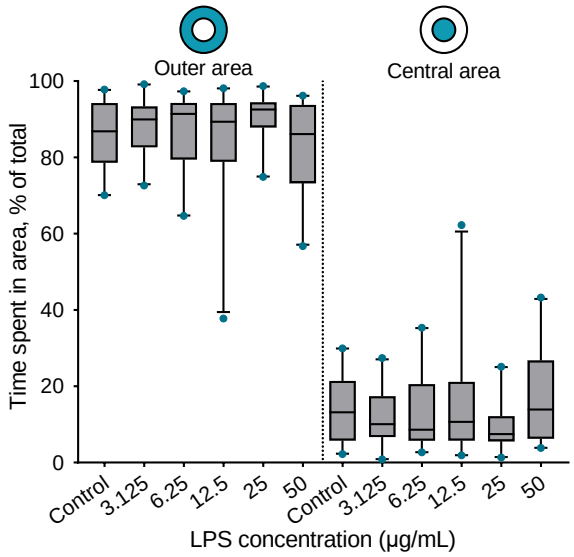
Table 2.7 – **Number of time points with statistically significant difference compared to control for data in Fig. 2.12.** Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of LPS. The statistical methods applied are outlined in the methods section (2.3.8). N=20 larvae per group.

	Control *vs.* 6.25 µg/mL	Control *vs.* 12.5 µg/mL	Control *vs.* 25 µg/mL	Control *vs.* 3.125 µg/mL	Control *vs.* 50 µg/mL
Time points with statistically significant difference *c*ontrol	0	0	0	1	1



(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Time spent by larvae in the central and outer area of the well as a percent of total observed time

Figure 2.13 – Assessment of behavioural differences in wild-type larval zebrafish after 48h of exposure to increasing doses of LPS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and time spent in the outer and central area of the well (c) were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=20 larvae per group. Total distance travelled (a), average speed (b) and thigmotaxis (edge preference) (c) were not significantly affected by LPS at these concentrations.

A new experiment was designed, with the same parameters as the previous study but with exposure concentrations of 25, 50 and 100 µg/mL for 24h, from 3 to 4 dpf. No mortality or morphological changes were observed at these concentrations in any exposure group. There was no apparent effect of LPS on dark-light transition response (Figure 2.14, Table 2.8). Other behavioural endpoints, such as total distance travelled, speed and thigmotaxis were not significantly affected (Figure 2.15). While not significant (p=0.0668), there was an increase in total distance travelled by larvae exposed to 100 µg/mL *vs* control larvae (Figure 2.15a).

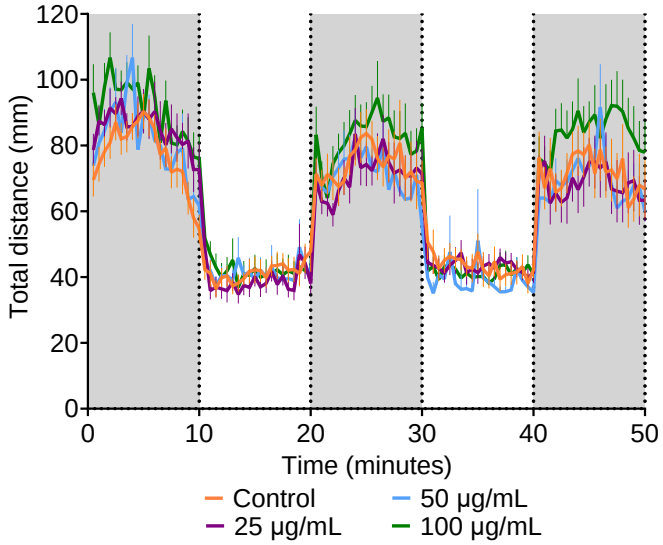
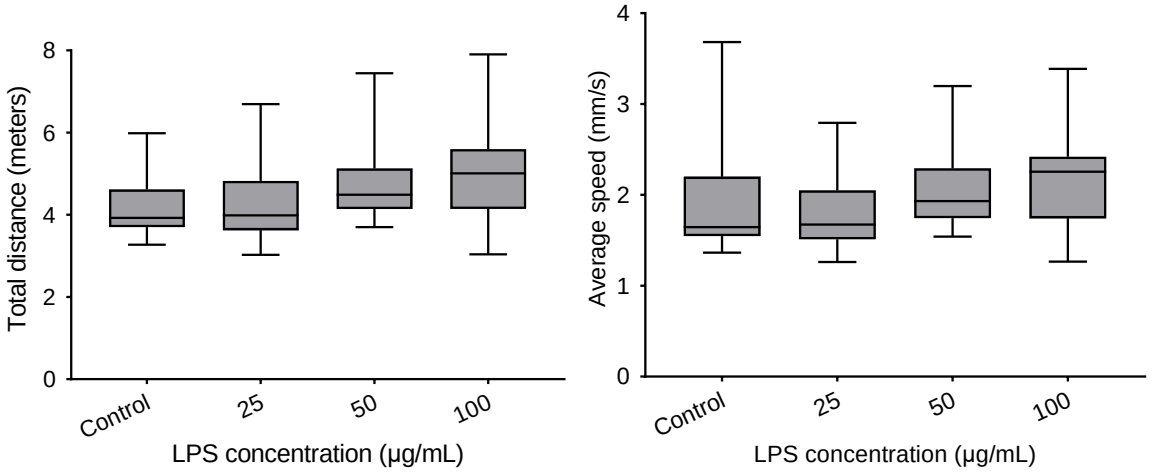


Figure 2.14 – Effect of alternating light-dark periods on locomotion in wild-type larval zebrafish after 24h of exposure to increasing doses of LPS. Larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=18 larvae per group. Exposure to 25 to 100 µg/mL LPS for 24h has no effect on the response of larvae to light and dark.

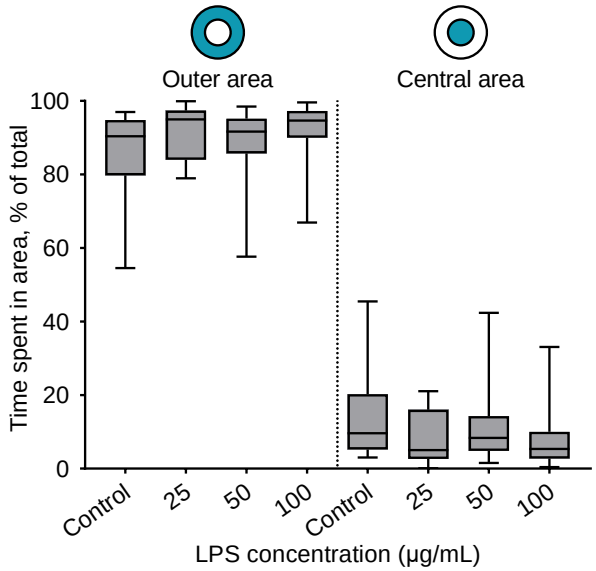
Table 2.8 – Number of time points with statistically significant difference compared to control for data in Fig. 2.14. Data represent the response to alternating light-dark periods on locomotion after 24h of exposure to increasing doses of LPS. The statistical methods applied are outlined in the methods section (2.3.8). N=18 larvae per group.

	Control vs. 25 µg/mL	Control vs. 50 µg/mL	Control vs. 100 µg/mL
Time points with statistically significant difference *c*ontrol	0	1	5



(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Time spent by larvae in the central and outer area of the well as a percent of total observed time

Figure 2.15 – Assessment of behavioural differences in wild-type larval zebrafish after 24h of exposure to increasing doses of LPS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and time spent in the outer and central area of the well (c) were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=28 larvae per group. While total distance travelled (a), average speed (b) and thigmotaxis (edge preference) (c) were not significantly affected by LPS, there seems to be an increase in total distance travelled by larvae exposed to 100 µg/mL *vs* control larvae (p=0.0668).

We hypothesise that the difference between these results *vs.* LPS exposures reported in the literature, which make it difficult to determine a range of concentrations that cause mortality or alter behaviour, can be explained by two factors: (1) previously

reported variability between batches and strains of LPS (Mottaz et al., 2017); (2) lack of absorption of LPS from water, given that the majority of experiments expose zebrafish through injection.

As defined in the approach set at the start of this project for selecting pro-inflammatory chemicals, only compounds capable of inducing behavioural changes with no evident toxicological effects were chosen for additional exposures to be conducted with Tg(MPx GFP i114) zebrafish larvae. Due to the lack of significant results at this stage, questions surrounding the uptake of LPS through water, and potential variability between batches, LPS was excluded from the subsequent experimental phase.

2.4.2.3 Copper Sulfate

Copper acts as a catalytic co-factor for various enzymes involved in energy and antioxidant metabolism (Linder and Hazegh-Azam, 1996). Exposure to high levels of inorganic copper can disturb the copper balance in zebrafish and lead to an inflammatory response that is mediated by damage from oxidative stress (Pereira et al., 2016).

While in adult zebrafish, copper sulfate ($CuSO_4$) was reported to induce oxidative damage and apoptosis in the gills and showed dose-dependent lethality (Craig et al., 2007; Griffitt et al., 2007), zebrafish larvae exposed to $CuSO_4$ display an inflamed status related to exacerbated damage and oxidative stress (Leite et al., 2013), activated neutrophils in the kidney marrow (Singh et al., 2014), and inhibited survival and development (Dave and Xiu, 1991; Johnson et al., 2007).

Previous studies reported that exposure of zebrafish larvae at 56 hpf to 10 µM $CuSO_4$ for 2 hours led to a general dispersal immune cells, suggestive of active migration from their initial location, with leukocytes specifically clustering around damaged neuromasts, small clusters of mechanosensory hair cells enclosed within a compact group of accessory cells. This damage is rapidly regenerated, and neuromasts recover full functionality one day after the damaging agent is removed (d'Alençon et al., 2010). Waterborne exposure of larvae to $CuSO_4$ has also been reported to cause lesions to superficial tissues followed by specific inflammatory responses at the sites of damage. Zebrafish larvae exposed to 10 µM $CuSO_4$ for up to 7 days displayed a general dispersal of leukocytes compared to controls and, in addition to lateral line neuromasts,

leukocyte infiltration was observed in the gills and nose (d'Alençon et al., 2010).

This accumulation of neutrophils in the neuromasts is one of the most frequently used indicators for the level of inflammation in this model and has been applied to assess the effect of known anti-inflammatory drugs. Furthermore, the induction of inflammation by $CuSO_4$ can easily be triggered by exposure to the compound in the culture medium (d'Alençon et al., 2010; Wittmann et al., 2012). Based on these studies with 10 µM $CuSO_4$, the selected exposure concentrations for the MTC experiment were 0.5, 2.5, 5, 25 and 50 µM $CuSO_4$.

At 68 hpf, after hatching, larvae were loaded into 24-well plates containing the selected concentrations of $CuSO_4$ in recirculating system water, along with a water-only control. A total of 144 larvae were used, with 24 larvae per exposure group.

After 48h exposure to $CuSO_4$, LC20 and LC50 values were calculated at 3.77 and 5.19 µM, respectively, using nonlinear regression analysis fitted to the data with Hill equation (Figure 2.16). While no mortality was observed at 0.5 µM $CuSO_4$, exposure to 25 µM or higher of $CuSO_4$ caused 100% mortality. No morphological changes were observed in the surviving exposure groups.

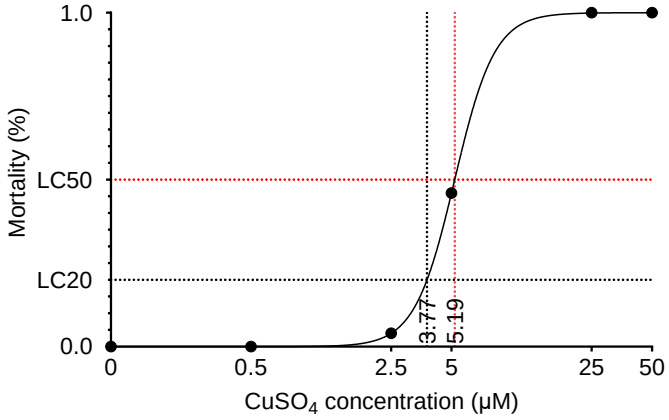


Figure 2.16 – Mortality of zebrafish after 48h exposure to $CuSO_4$ at different concentrations. Larvae were introduced to the dilutions from 68 hpf. N=24 larvae per group. LC20 and LC50 values, were calculated using nonlinear regression analysis fitted to the data with Hill equation.

Locomotion and dark-light transition response were increasingly disrupted in all surviving exposure groups, dependent on concentration (Figure 2.17, Table 2.9). This response can also be observed by a significant decrease in distance travelled and

average speed in zebrafish larvae exposed to 2.5 and 5 µM $CuSO_4$. Thigmotactic behaviour was not significantly altered in any exposure group.

As defined in the approach set at the start of this project for selecting pro-inflammatory chemicals, concentrations capable of inducing behavioural changes with no evident toxicological effects were chosen for additional exposures to be conducted with Tg(MPx GFP i114) zebrafish larvae. As the concentrations selected aim to minimise the risk of inducing mortality, further experiments exposed zebrafish to 0.1, 0.5, and 2.5 µM $CuSO_4$, testing both behaviour and infiltration of neutrophils in the gut.

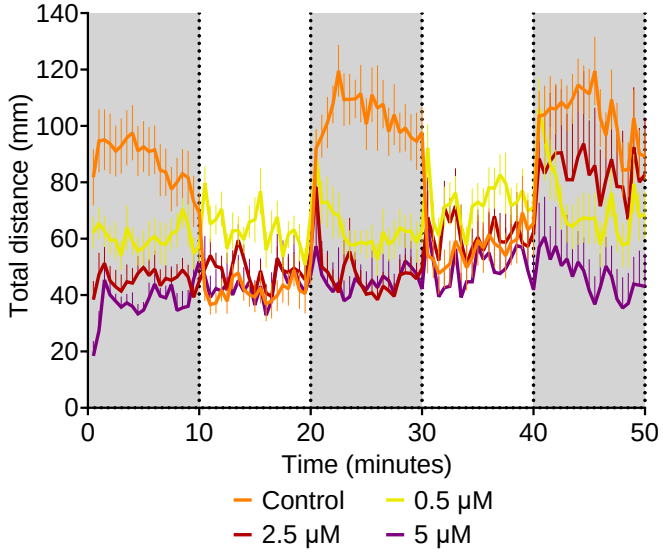
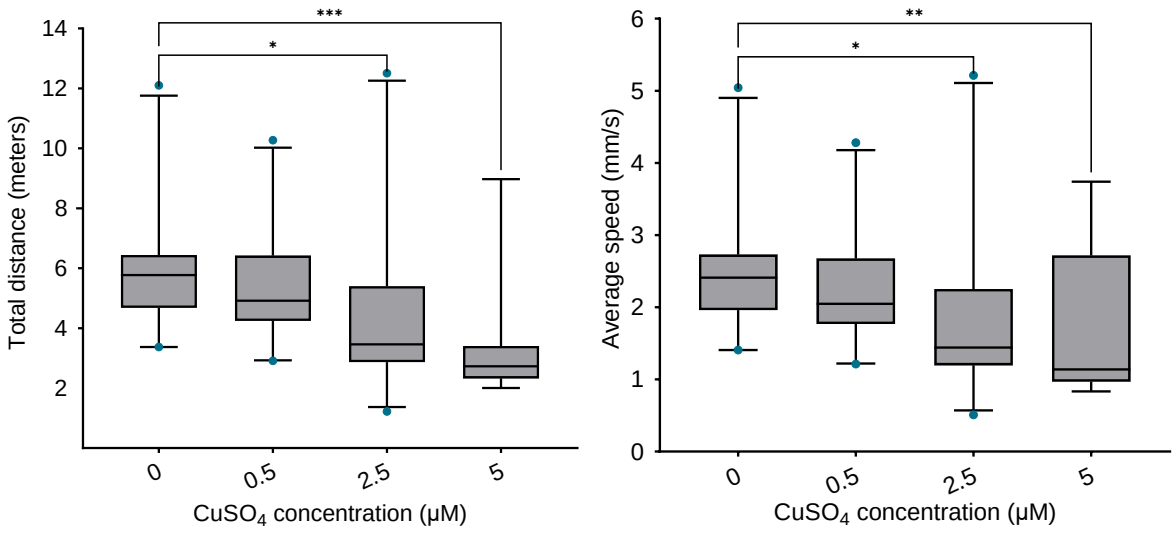


Figure 2.17 – Effect of alternating light–dark periods on locomotion in wild-type larval zebrafish after 48h of exposure to increasing doses of $CuSO_4$. Larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=23 (Control, 0.5 µM), 22 (2.5 µM) and 11 (5 µM) larvae per group. Exposure to $CuSO_4$ ranging from 0.5 to 5 µM increasingly disrupts locomotion and response of larvae to light and dark.

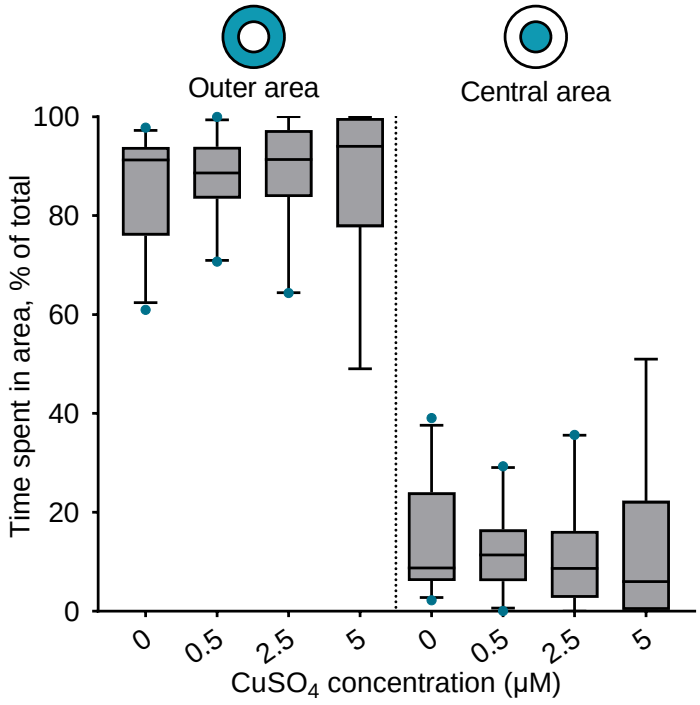
Table 2.9 – Number of time points with statistically significant difference compared to control for data in Fig. 2.17. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of $CuSO_4$. The statistical methods applied are outlined in the methods section (2.3.8). N=23 (Control, 0.5 µM), 22 (2.5 µM) and 11 (5 µM) larvae per group.

	Control vs. 0.5 µM	Control vs. 2.5 µM	Control vs. 5 µM
Time points with statistically significant difference *c*ontrol	42	40	56



(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Time spent by larvae in the central and outer area of the well as a percent of total observed time

Figure 2.18 – Assessment of behavioural differences in wild-type larval zebrafish after 48h of exposure to increasing doses of $CuSO_4$. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and time spent in the outer and central area of the well (c) were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=23 (Control, 0.5 µM), 22 (2.5 µM) and 11 (5 µM) larvae per group. Total distance travelled (a) and average speed (b) significantly decreased in zebrafish larvae exposed to 2.5 and 5 µM $CuSO_4$. Thigmotaxis (edge preference) (c) was not significantly altered in any exposure group.

2.4.2.4 DSS

DSS is a synthetic sulfated branched polysaccharide derivative of dextran, widely used on mice and rats in studies of IBD pathogenesis (Mizoguchi, 2012; Hanyang et al., 2017). In zebrafish, DSS immersion has successfully been used to induce intestinal inflammation (Oehlers et al., 2013, 2017; Chuang et al., 2019), resulting in elevated expression of pro-inflammatory genes and neutrophil recruitment around the intestine (Oehlers et al., 2012). The most accepted mechanism of DSS-induced intestinal inflammation is the disruption of the intestinal epithelial monolayer lining, leading to the entry of luminal bacteria and associated antigens into the mucosa and allowing the dissemination of pro-inflammatory intestinal contents into underlying tissue (Kiesler et al., 2015; Perše and Cerar, 2012; Wirtz et al., 2007; Okayasu et al., 1990; Poritz et al., 2007; Kim et al., 2012; Samak et al., 2015; Eichele and Kharbanda, 2017). This is also associated with increased production of various cytokines and chemokines as early as the first day of DSS-induction, including TNF-α, the hallmark of DSS-induced colitis, IL-6, IL-10, IL-17, IL-1β, transforming growth factor beta (TGF-β), mucin, TLR2/4 gene expression, and MPO activity (Randhawa et al., 2014; Perše and Cerar, 2012).

Doses typically utilised in murine models (2%–5% (w/v)) administered *ad libitum* in the drinking water were found to be highly toxic to larval zebrafish resulting in rapid mortality (Oehlers et al., 2013). Exposure to 0.5% (w/v) DSS has routinely been used to induce inflammation and intestinal mucus accumulation in 5 dpf zebrafish larvae, while 0.25% (w/v) reportedly does not induce significant visible inflammation but still induces protective intestinal mucus accumulation (Oehlers et al., 2013, 2017, 2012). At doses above 0.6% (w/v), larval mortality rates have been reported to be variable, resulting in either complete cohort survival or mortality within 2 days of exposure (Oehlers et al., 2013). However, it has also been reported that 0.25% and 0.1% (w/v) DSS induces dose-dependent mortality with repeated injury (68% and 29%, respectively) in zebrafish larvae exposed from 5 to 12 dpf (Chuang et al., 2019). Based on these studies, the selected exposure concentrations for the MTC experiment were 2.25, 4.5, and 9 mg/mL of DSS in water.

At 68 hpf, after hatching, larvae were loaded into 24-well plates containing the

selected concentrations of TNBS in recirculating system water, along with a water-only control. A total of 144 larvae were used, with 24 larvae per exposure group.

After 48h exposure, mortality occurred in all exposure groups, with the highest mortality rates at 9 mg/mL (95.4%). At the lowest exposure dose, 2.25 mg/mL, the mortality rate was 22.7%. The LC20 and LC50 values were calculated at 2.22 and 3.74 mg/mL DSS, respectively, using nonlinear regression analysis fitted to the data with the Hill equation (Figure 2.19).

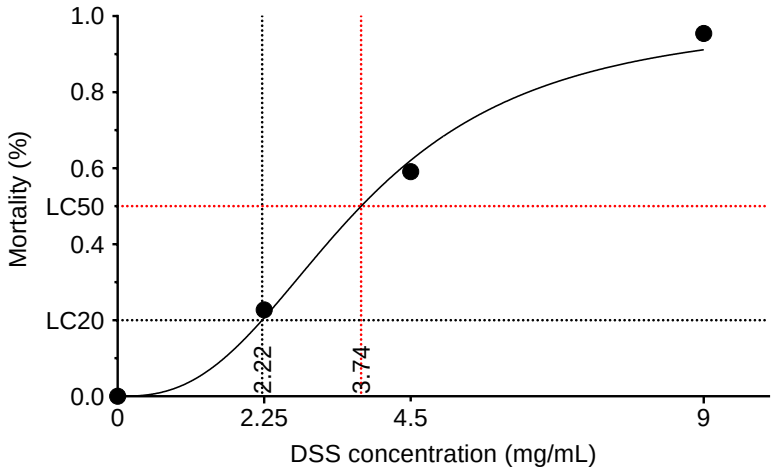
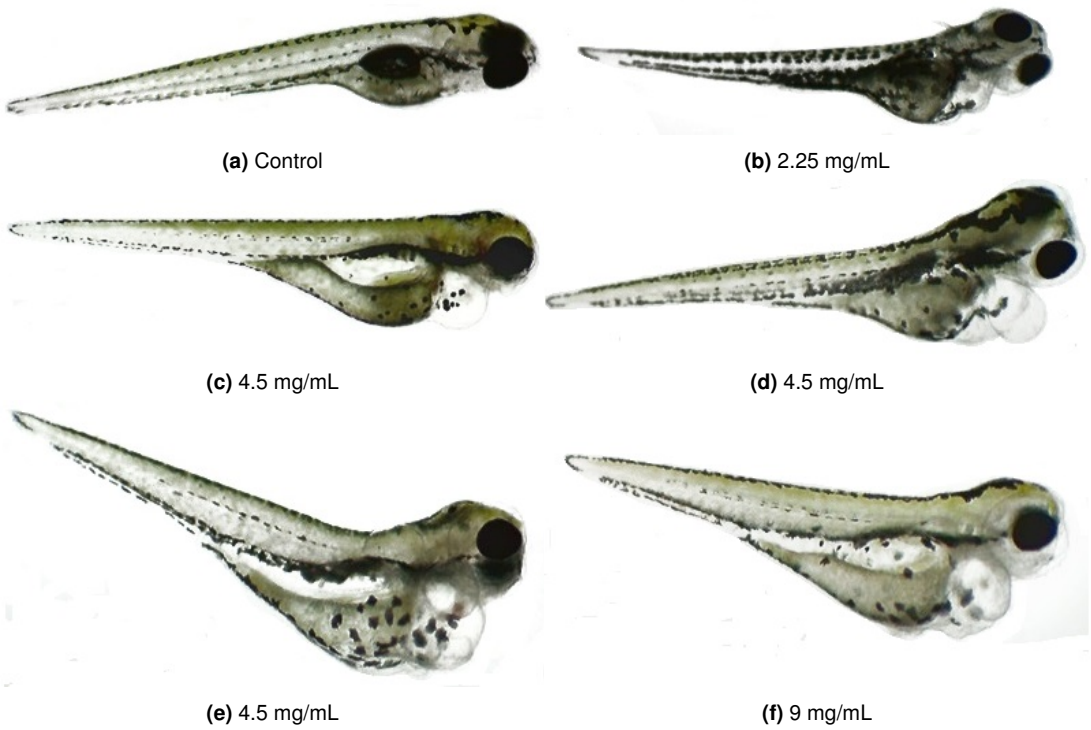


Figure 2.19 – **Mortality of zebrafish after 48h exposure to DSS at different concentrations.** Larvae were introduced to the dilutions from 68 hpf. N=22 larvae per group. LC20 and LC50 values, were calculated using nonlinear regression analysis fitted to the data with Hill equation.

High percentages of posture abnormalities were observed in all concentrations. After 24h exposure to DSS, over 80% of the larvae in all exposure concentrations were still moving, but on their side or upside down. Larvae exposed to 4.5 mg/mL were twitching, and most demonstrated impaired movement. After 48h exposure, 87% of larvae exposed to 2.25 mg/mL were on their side or upside down, while 100% of remaining living larvae exposed to 4.5 and 9 mg/mL displayed the same difficulties. In the two higher concentrations, larvae consistently showed deflated swim bladders as well as gut, yolk sac and pericardial oedema, which explains the swimming difficulties and points to toxicity in all exposure concentrations (Fig. 2.20).

Dark-light transition response was disrupted in zebrafish larvae exposed to DSS (Table 2.10, with any response to dark and light stimuli no longer present (Figure 2.21). While overall locomotion was not affected in larvae exposed to 4.5 mg/mL DSS, larvae



(a) Control **(b)** 2.25 mg/mL

(c) 4.5 mg/mL **(d)** 4.5 mg/mL

(e) 4.5 mg/mL **(f)** 9 mg/mL

Figure 2.20 – High percentages of posture abnormalities and swimming difficulties in zebrafish larvae caused by exposure to 2.5 to 9 mg/mL of DSS. Exposure to 4.5 and 9 mg/mL DSS consistently presented with deflated swim bladders as well as gut, yolk sac and pericardial oedema.

exposed to 2.25 mg/mL DSS displyed a significant increase in distance travelled and average speed versus controls (Figures 2.22a and 2.22b). However, the lack of response in larvae exposed to 4.5 mg/mL can be explained by the higher mortality rates and posture deformities, which affected the movement of some larvae and, therefore, the locomotion test. The final sample size after mortality in this exposure group was only 6 larvae, making the data for this exposure group very scattered and unreliable. Thigmotactic behaviour was not significantly altered in any exposure group (Figure 2.22c).

As defined in the approach set at the start of this project for selecting pro-inflammatory chemicals, concentrations capable of inducing behavioural changes with no evident toxicological effects were chosen for additional exposures to be conducted with Tg(MPx GFP i114) zebrafish larvae. As the concentrations selected aim to minimise the risk of inducing mortality, further experiments exposed zebrafish to concentrations below 2.25 mg/mL (77.3% survival rate): 0.65 mg/mL, 1.25, and 2.5 mg/mL, testing both behaviour and infiltration of neutrophils in the gut.

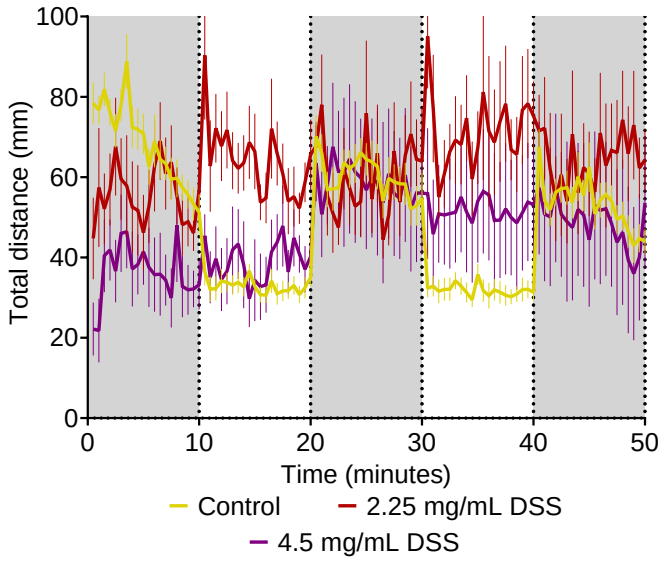
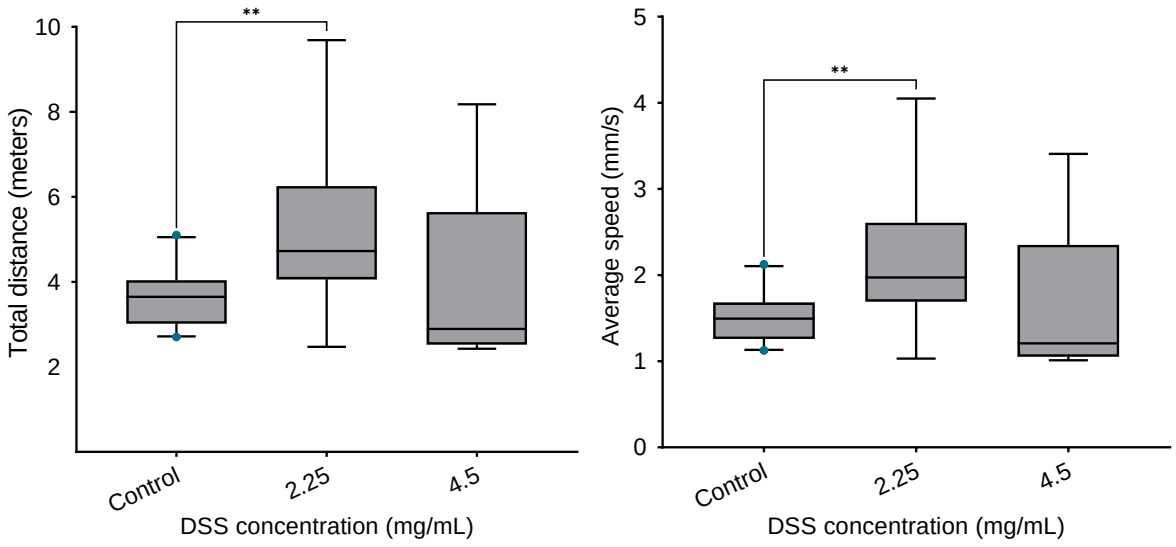


Figure 2.21 – Effect of alternating light–dark periods on locomotion in wild-type larval zebrafish after 48h of exposure to 0.25 and 4.5 mg/mL DSS. Larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=23 (Control), 13 (2.25 mg/mL) and 6 (4.5 mg/mL) larvae per group. Exposure to 2.25 and 4.5 mg/mL DSS disrupts the response of larvae to light and dark, although overall locomotion appears to increase in larvae exposed to 2.25 mg/mL.

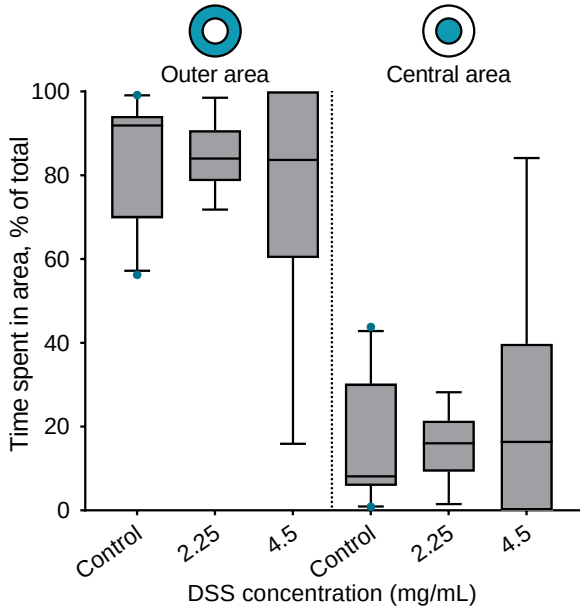
Table 2.10 – Number of time points with statistically significant difference compared to control for data in Fig. 2.21. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of $CuSO_4$. The statistical methods applied are outlined in the methods section (2.3.8). N=23 (Control), 13 (2.25 mg/mL) and 6 (4.5 mg/mL) larvae per group.

	Control vs. 0.5 µM	Control vs. 2.5 µM	Control vs. 5 µM
Time points with statistically significant difference *c*ontrol	42	40	56



(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Time spent by larvae in the central and outer area of the well as a percent of total observed time

Figure 2.22 – Assessment of behavioural differences in wild-type larval zebrafish after 48h of exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and and time spent in the outer and central area of the well (c) were plotted. data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=23 (Control), 13 (2.25 mg/mL) and 6 (4.5 mg/mL) larvae per group. Total distance travelled (a) and average speed (b) significantly increased in zebrafish larvae exposed to 2.25 mg/mL DSS. Thigmotaxis (edge preference) (c) was not significantly altered in any exposure group.

2.4.3 Phase Two: 48-hour exposures

2.4.3.1 Behaviour and immune profile of zebrafish larvae exposed to 2,4,6-trinitrobenzene sulfonic acid (TNBS) for 48 hours

Following the results of the MTC experiment, a new exposure was conducted with 25, 50 and 100 µg/mL TNBS. The study was conducted through two independent experiments that spanned over a period of two days. At 68 hpf, after hatching, larvae were loaded into each well of a 24-well plate containing the selected concentrations of TNBS in recirculating system water, along with a water-only control. A total of 164 larvae were used, with 41 larvae per exposure group. Zebrafish larvae were maintained as described in Section 2.3. After 48h exposure, no morphological changes or mortality were observed in any exposure group.

As previously demonstrated in the MTC study, the administration of TNBS did not result in any discernible effect on dark-light transition response in any exposure groups. However, overall locomotion decreased with increasing concentrations of TNBS (Fig. 2.23, Table 2.11). This can also be seen in the total distance travelled (Fig. 2.24a) and speed of larvae (Fig. 2.24b), both of which are significantly lower in larvae exposed to 50 and 100 µg/mL of TNBS when compared to controls. Larvae exposed to concentrations of 50 and 100 µg/mL TNBS exhibited predominantly reduced motility, characterized by a significant decrease in their velocity profile, with a notable proportion of the motion attributed to drifting (Fig. 2.24c).

Thigmotaxis was not significantly affected in larvae exposed to TNBS (Fig. 2.25). Exposure to 50 and 100 µg/mL TNBS significantly increased the number of neutrophils in the mid and posterior gut of zebrafish larvae (Fig. 2.26).

Both behaviour and immune response were significantly altered in zebrafish larvae exposed to 50 and 100 µg/mL, making TNBS a candidate for further testing. However, although TNBS showed promising results in inducing modulation of behaviour as well as inflammation, different experiments performed throughout the project generated conflicting results. Given the absence of changes in both light and edge preferences, the behavioural disruptions observed during this stage are likely attributable to compound toxicity, independent of any inflammatory responses.

Furthermore, TNBS is reported to be highly reactive with reducing agents (Merck, 2019), while all selected pharmaceutical drugs and treatments intended to be used in combination with TNBS are incompatible with strong oxidising agents. This reaction was clearly visible during the preparation for imaging when larvae were transferred from 100 µg/mL TNBS medium to MS-222. Prolonged exposure of the larvae to 100 mg/L MS-222 resulted in severe deformation of the spine, and a discernible colour change of the transparent medium to yellow occurred within 30 minutes. For these reasons, TNBS was excluded as a candidate for further testing.

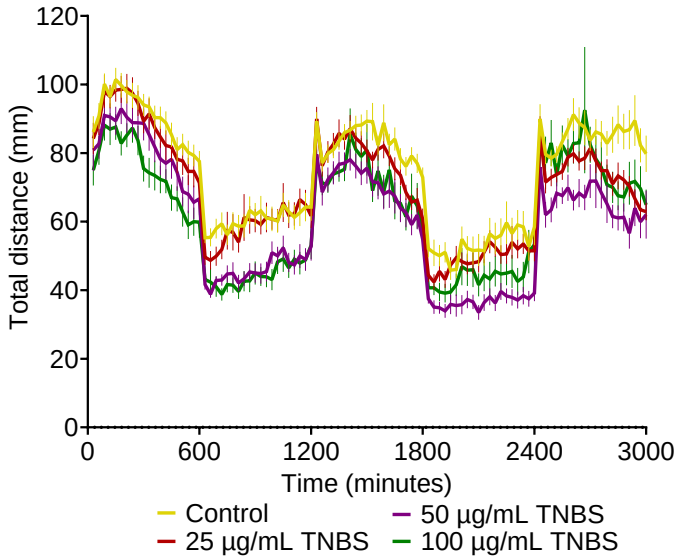
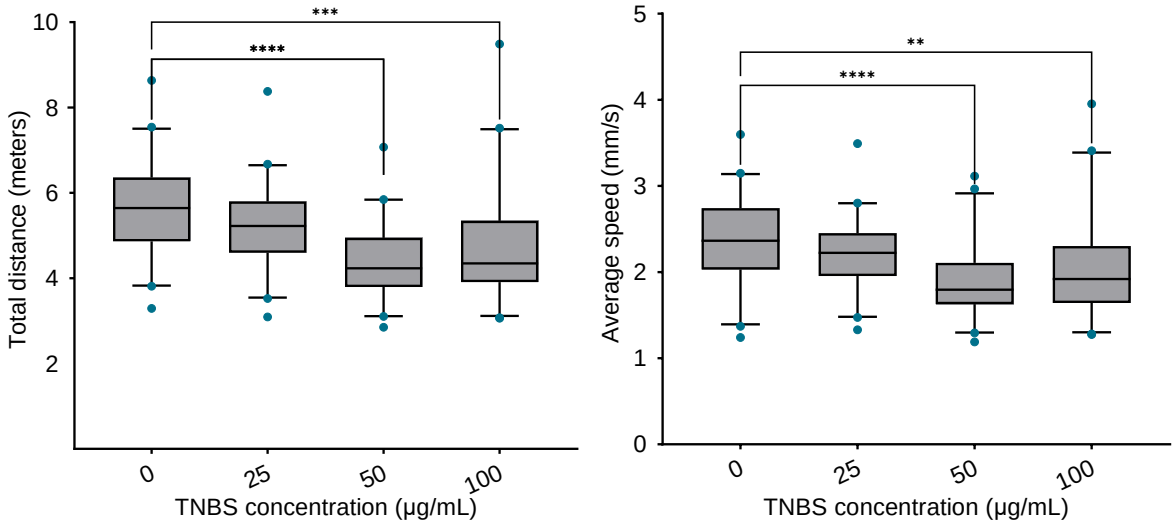


Figure 2.23 – Effect of alternating light–dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of TNBS. Free swimming hatched larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=41 larvae per group. Exposure to 25 to 100 µg/mL of TNBS has no effect on the response of larvae to light and dark, but causes a slightly decrease in locomotion.

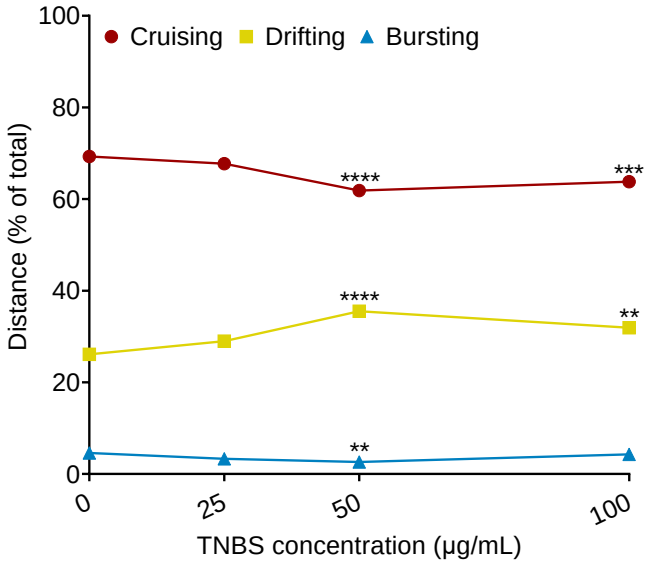
Table 2.11 – Number of time points with statistically significant difference compared to control for data in Fig. 2.23. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of TNBS. The statistical methods applied are outlined in the methods section (2.3.8). N=41 larvae per group.

	Control vs. 25 µg/mL	Control vs. 50 µg/mL	Control vs. 100 µg/mL
Time points with statistically significant difference *vs.* control	7	63	42



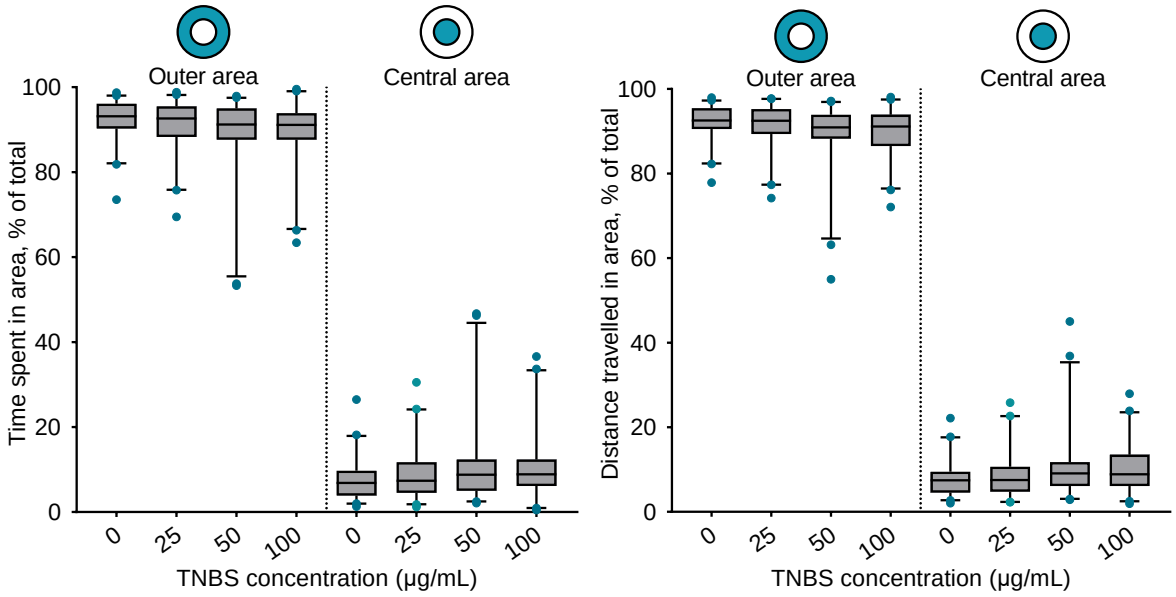
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Distance travelled at different speed thresholds

Figure 2.24 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of TNBS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=41 larvae per group. Exposure to 50 and 100 µg/mL TNBS significantly lowers total distance travelled (a) and average speed (b) *vs* controls. At these concentrations, a significantly higher proportion of distance travelled can be attributed to drifting (c).



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well as a percent of total distance travelled

Figure 2.25 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of TNBS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=41 larvae per group. Exposure to 25, 50 and 100 µg/mL TNBS did not significantly alter thigmotaxis (edge preference) *vs.* controls.

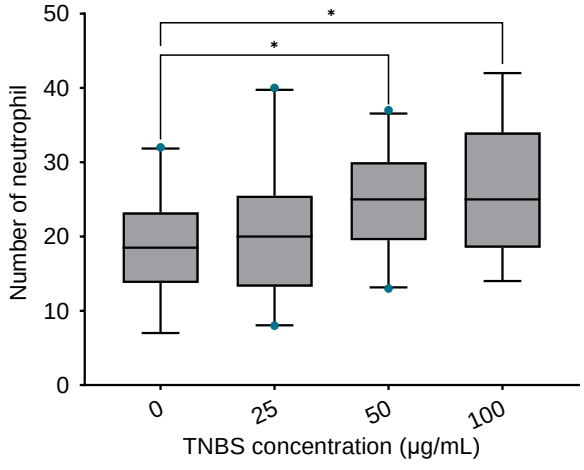


Figure 2.26 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (as demarcated in Fig. 2.4) in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of TNBS. Larvae were introduced to the dilutions from 68 hpf. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=41 larvae per group. N=22 (Control and 50 µg/mL), 20 (25 µg/mL) and 17 (100 µg/mL) larvae per group. Exposure to 50 and 100 µg/mL TNBS significantly increases the number of neutrophils in the gut *vs.* controls.

2.4.3.2 Behaviour and immune profile of zebrafish larvae exposed to Copper Sulfate for 48 hours

Following the results of the MTC experiment, an additional exposure was conducted with 0.1, 0.5 and 2.5 µM $CuSO_4$. The study was conducted through two independent experiments that spanned over a period of two days. At 68 hpf, after hatching, larvae were loaded into each well of a 24-well plate containing the selected concentrations of TNBS in recirculating system water, along with a water-only control. A total of 192 larvae were used, with 48 larvae per exposure group. Zebrafish larvae were maintained as described in Section 2.3. After 48h exposure, no morphological changes or mortality were observed in any exposure group.

Our results indicated that $CuSO_4$ had a minor impact on dark-light transition response across all exposure groups, which was consistent with previous research conducted in the MTC study (Fig. 2.27, Table 2.12). However, an unexpected observation was made in some larvae groups, including the control group, from the 30-minute marker onwards. These groups displayed a lack of response to light stimuli, indicating impaired dark-light transition response. This phenomenon was observed across all plates in the first independent experiment, as demonstrated in the accompanying graphs in Annex C, but was not observed in the second set of plates recorded.

Overall locomotion decreases significantly compared to controls in larvae exposed to 0.1 and 0.5 µM $CuSO_4$, as seen in the total distance travelled (Fig. 2.28a) and average speed of larvae (Fig. 2.28b). Exposure of larvae to 2.5 µM $CuSO_4$ resulted in a reduction in both total distance traveled and average speed, but these differences were not statistically significant ($p=0.1295$ and $p=0.1282$, respectively). Larvae exposed to 0.5 and 2.5 µM also travel significantly less at cruising speeds, with more of the movement being attributed to drifting and bursting speeds (Fig. 2.28c).

Although there was no difference in the time spent in the outer *vs* the central area of the well, larvae exposed to 0.5 µM $CuSO_4$ displayed a significant preference for the central area of the well when compared to controls (Fig. 2.29).

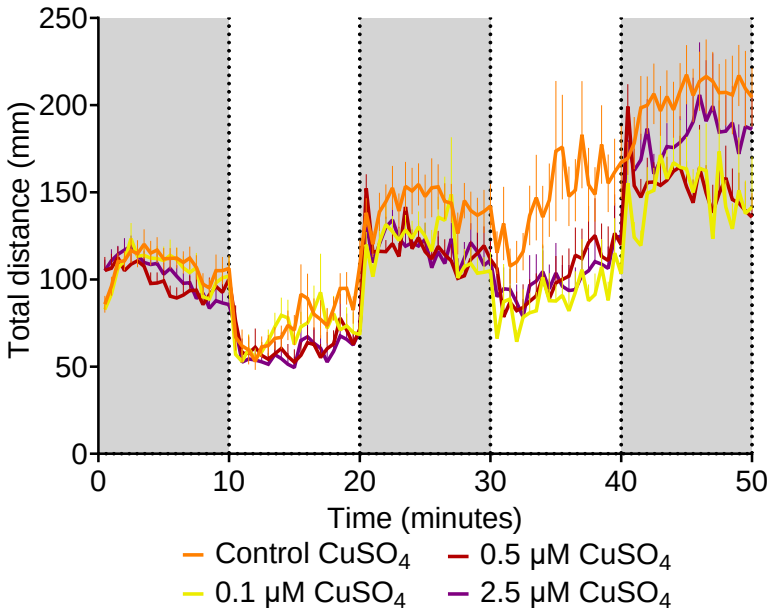
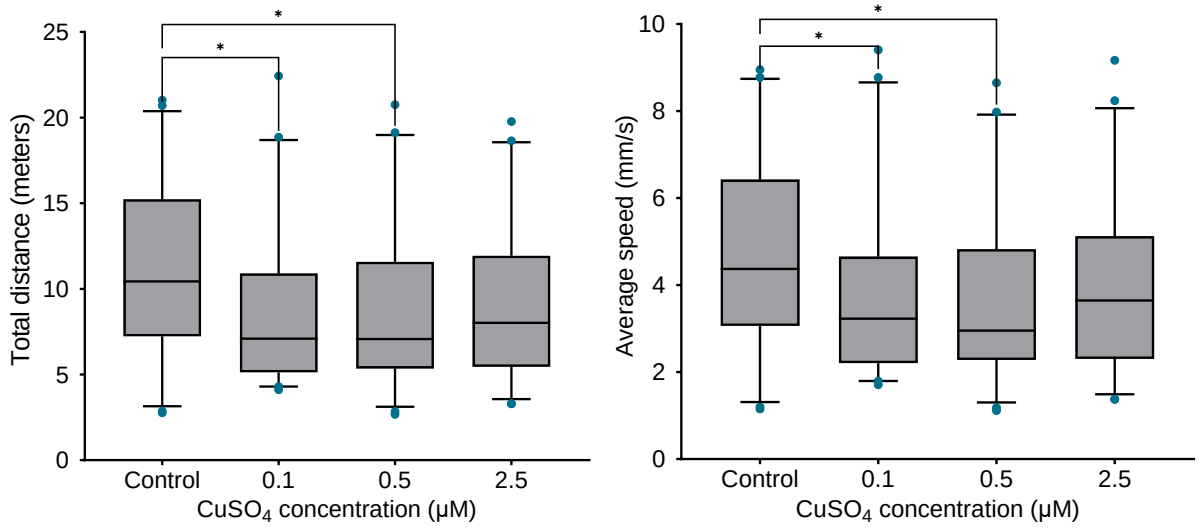


Figure 2.27 – Effect of alternating light–dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of $CuSO_4$. Free swimming hatched larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=44 (Control), 40 (0.1 µM) and 45 (0.5 and 2.5 µM) larvae per group. Exposure to $CuSO_4$ ranging from 0.1 to 2.5 µM has no effect the response of larvae to light and dark, but causes a slightly decrease in locomotion.

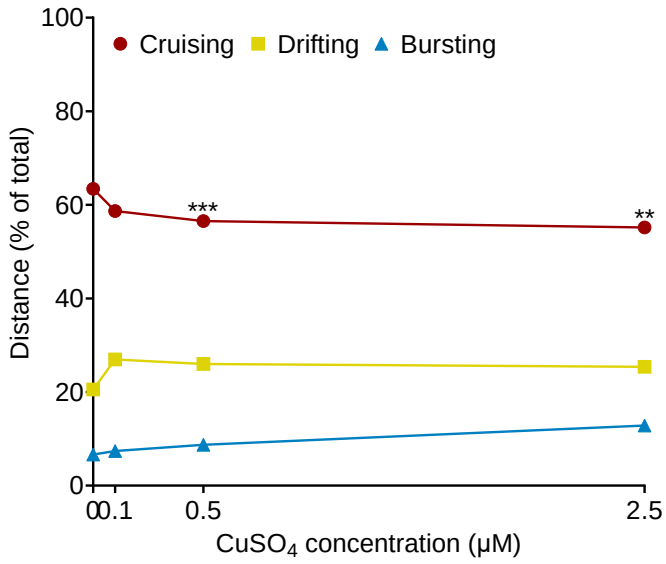
Table 2.12 – Number of time points with statistically significant difference compared to control for data in Fig. 2.27. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of $CuSO_4$. The statistical methods applied are outlined in the methods section (2.3.8). N=44 (Control), 40 (0.1 µM) and 45 (0.5 and 2.5 µM) larvae per group.

	Control vs. 0.1 µM	Control vs. 0.5 µM	Control vs. 2.5 µM
Time points with statistically significant difference *vs.*control	32	27	13



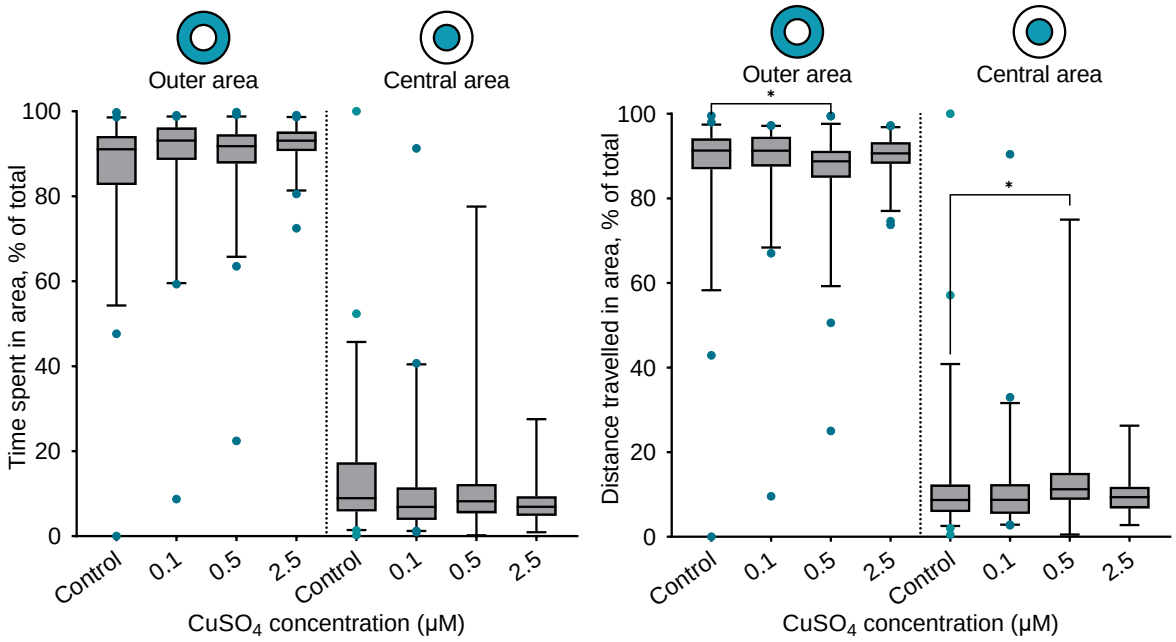
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Distance travelled at different speed thresholds

Figure 2.28 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of $CuSO_4$. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=44 (Control), 40 (0.1 µM) and 45 (0.5 and 2.5 µM) larvae per group. Exposure to 0.1 and 0.5 µM $CuSO_4$ significantly lowers total distance travelled (a) and average speed (b) *vs* controls. For larvae exposed to 0.5 and 2.5 µM $CuSO_4$, a significantly lower proportion of distance travelled can be attributed to cruising, with and increase in movements at drifting and bursting speed(c).



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well as a percent of total distance travelled

Figure 2.29 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of $CuSO_4$. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=44 (Control), 40 (0.1 µM) and 45 (0.5 and 2.5 µM) larvae per group. The distance travelled in the outer area was decreased in larvae exposed to 0.1 and 0.5 µM $CuSO_4$ (Fig. 2.29), while the distance travelled in the central area was also lower in larvae exposed to 0.1 µM $CuSO_4$

While the behavioural response alterations make $CuSO_4$ a candidate for further testing, the quantification of neutrophil infiltration presented challenges. As previously described, $CuSO_4$ exposure in zebrafish leads to a clustering of immune cells around the neuromasts, clusters of mechanosensory hair cells that comprise the zebrafish lateral line, a sensory system used to detect changes in water flow (Thomas et al., 2015). Neuromasts are located in stereotyped locations on the head, comprising the anterior lateral line system, or along the body, forming the posterior lateral line system (Fig. 2.30). Due to the location of the neuromasts, accurate quantification of neutrophil infiltration using manual methods presented significant difficulties. Firstly, due to the age of the zebrafish and the presence of not only a swim bladder but remnants of an egg sack, it was not possible to position the larvae top-up, to aid the visualisation of neutrophil accumulation in the neuromasts. When larvae were positioned sideways,

increased fluorescence was observed in the gut of larvae exposed to $CuSO_4$ (Fig. 2.31). However, due to the overlap of the fluorescent signals, it was impossible to count individual neutrophils manually. Future work to develop a method to quantify fluorescence in zebrafish larvae automatically could shed light on the effects of $CuSO_4$ in zebrafish larvae.

Although $CuSO_4$ shows promising results when it comes to inducing modulation of behaviour through inflammation, considering these difficulties and taking into consideration the favourable results obtained with DSS exposure, $CuSO_4$ has been eliminated as a candidate for further testing.

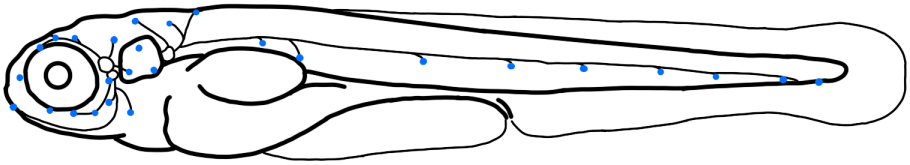
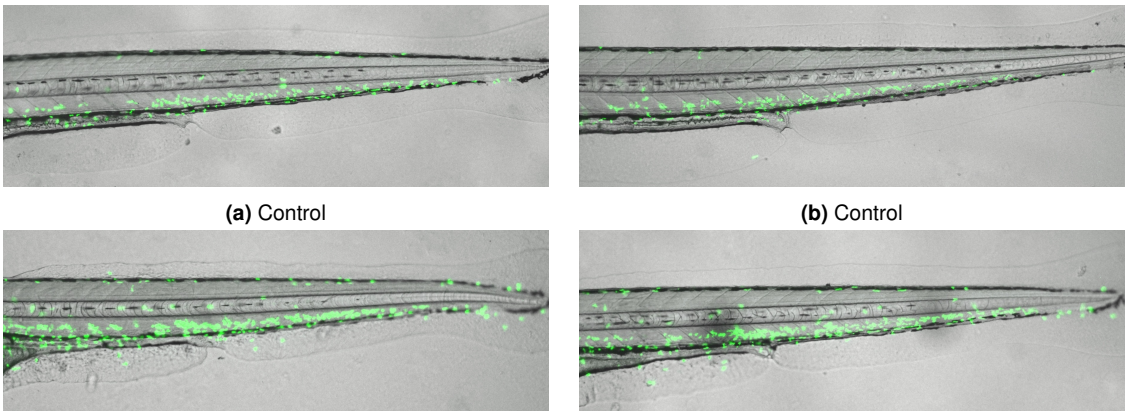


Figure 2.30 – Schematic of neuromast locations (blue dots) in a 5 dpf zebrafish larvae. Adapted from Whitfield



(a) Control **(b)** Control

(c) 2.5 µM **(d)** 2.5 µM

Figure 2.31 – Infiltrating neutrophils in the mid and posterior section of the gut in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to 2.5µM of $CuSO_4$. Larvae were introduced to the dilutions from 68 hpf. Exposure to DSS led an increase in fluorescent signals in the gut.

2.4.3.3 Behaviour and immune profile of zebrafish larvae exposed to Dextran Sulfate Sodium (DSS) for 48 hours

Following the results of the MTC experiment, a new exposure was conducted with 0.65, 1.25 and 2.5 mg/mL DSS. The study was conducted through two independent experiments that spanned over a period of two days. At 68 hpf, after hatching, larvae were loaded into each well of a 24-well plate containing the selected concentrations of TNBS in recirculating system water, along with a water-only control. A total of 84 larvae were used, with 21 larvae per exposure group. After 48h exposure to 2.5 mg/mL DSS, mortality rates were 27.3% (Figure 2.32). No mortality was observed at the other exposure concentrations. This is consistent with the results from the previous MTC study, where mortality rates for 3 dpf larvae exposed to 2.25 mg/mL DSS for 48h were 22.7%. No morphological changes were observed in any exposure group.

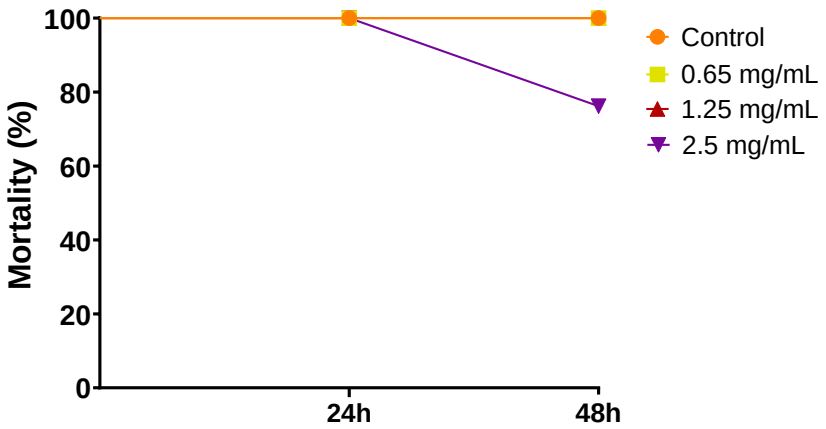


Figure 2.32 – Survival of zebrafish larvae after 48h of exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 68 hpf. N=21 larvae per group. Exposure to 2.5 mg/mL DSS resulted in 27.3% mortality. No mortality was observed in other exposure concentrations.

As previously demonstrated in the MTC study, DSS disrupts dark-light transition response in a dose-dependent manner in all exposure groups (Fig. 2.33, Table 2.13). Overall locomotion was decreased in larvae exposed to DSS, as demonstrated in the total distance travelled (Fig. 2.34a) which was significantly lower in larvae exposed to 1.25 and 2.5 mg/mL DSS compared to controls, and average speed of larvae exposed to 2.5 mg/mL DSS *vs* controls (Fig. 2.34b).

While exposure to 0.65 mg/mL seemed to increase the distance travelled and

average speed, this change was not significant (p=0.1041 and p=0.4371, respectively). Larvae exposed to 1.25 and 2.5 µg/mL DSS travel at lower speeds, with significantly less movement being attributed to bursting speeds (Fig. 2.24c), and higher distances travelled while drifting.

Thigmotaxis was affected in larvae exposed to $CuSO_4$. While the slightly increased preference for spending time in the central area in larvae exposed to 1.25 and 2.5 mg/mL was not statistically significant, larvae exposed to 1.25 mg/mL moved significantly more in the central area compared to the outer area of the well (Fig. 2.35).

Exposure to 1.25 and 2.5 mg/mL DSS significantly increases the number of neutrophils in the mid and posterior gut of zebrafish larvae (Fig. 2.36).

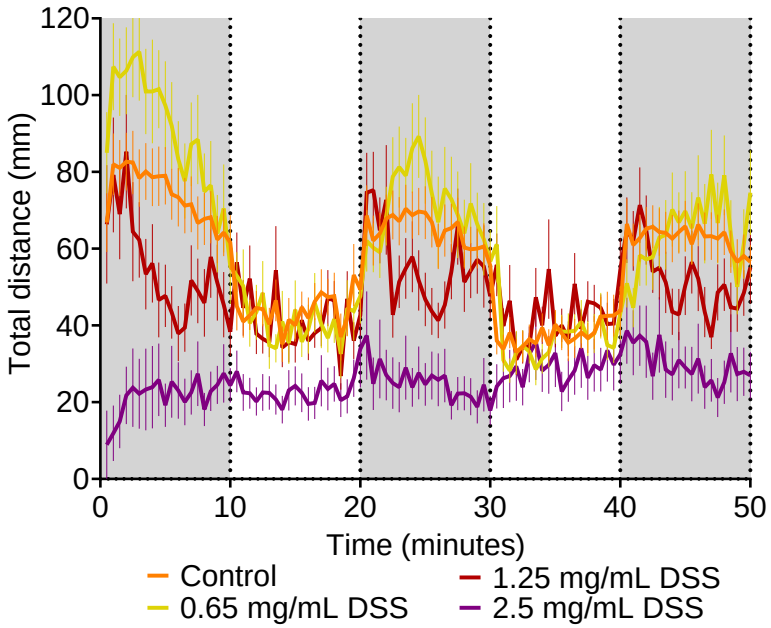
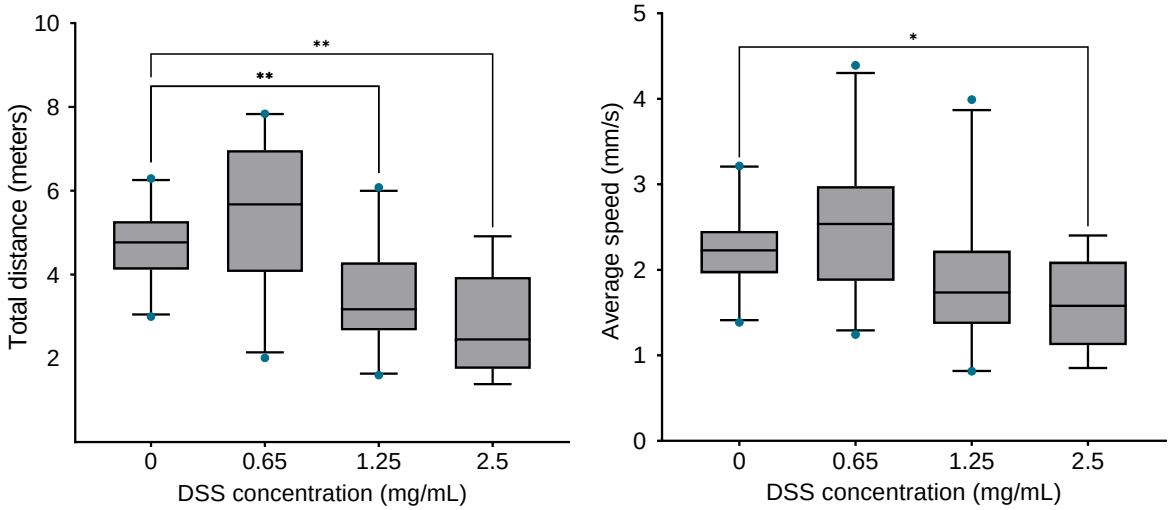


Figure 2.33 – Effect of alternating light-dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of DSS. Free swimming hatched larvae were introduced to the dilutions from 68 hpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=21 larvae per group, with the exception of 2.5mg/mL, where n=9 due to increased mortality rates. Exposure to 1.25 and 2.5 mg/mL DSS disrupts the response of larvae to light and dark and locomotion, although overall locomotion appears to increase in larvae exposed to 0.65 mg/mL.

Table 2.13 – Number of time points with statistically significant difference compared to control for data in Fig. 2.33. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). N=21 larvae per group, with the exception of 2.5mg/mL, where n=9 due to increased mortality rates.

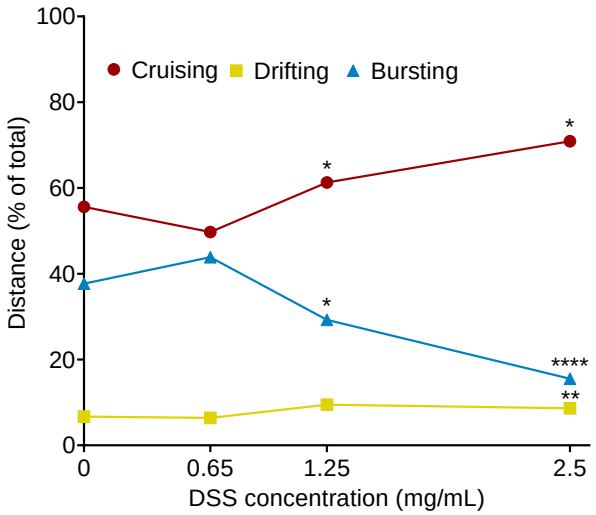
	Control vs. 0.65 mg/mL	Control vs. 1.25 mg/mL	Control vs. 2.5 mg/mL
Time points with statistically significant difference *vs.* control	4	8	60

Considering zebrafish display thigmotactic anxiety-related behaviour - they prefer to be on the edge of a Petri dish over the centre - the effect observed in larvae exposed to 1.25 mg/mL can be indicative of a behavioural alteration in the presence of DSS that the toxicity of the compound cannot explain. Both behaviour and immune response were significantly altered in zebrafish larvae exposed to 1.25 and 2.5 mg/mL, making DSS a candidate for further testing. As defined in the approach set at the start of this project for selecting pro-inflammatory chemicals, concentrations capable of inducing behavioural changes with no evident toxicological effects were chosen for longer 10-day exposures to be conducted with Tg(MPx GFP i114) zebrafish larvae. As the concentrations selected aim to minimise the risk of inducing mortality, further experiments exposed zebrafish to concentrations below 2.5 mg/mL (0.325, 1.65, and 1.3 mg/mL) testing both behaviour and infiltration of neutrophils in the gut.



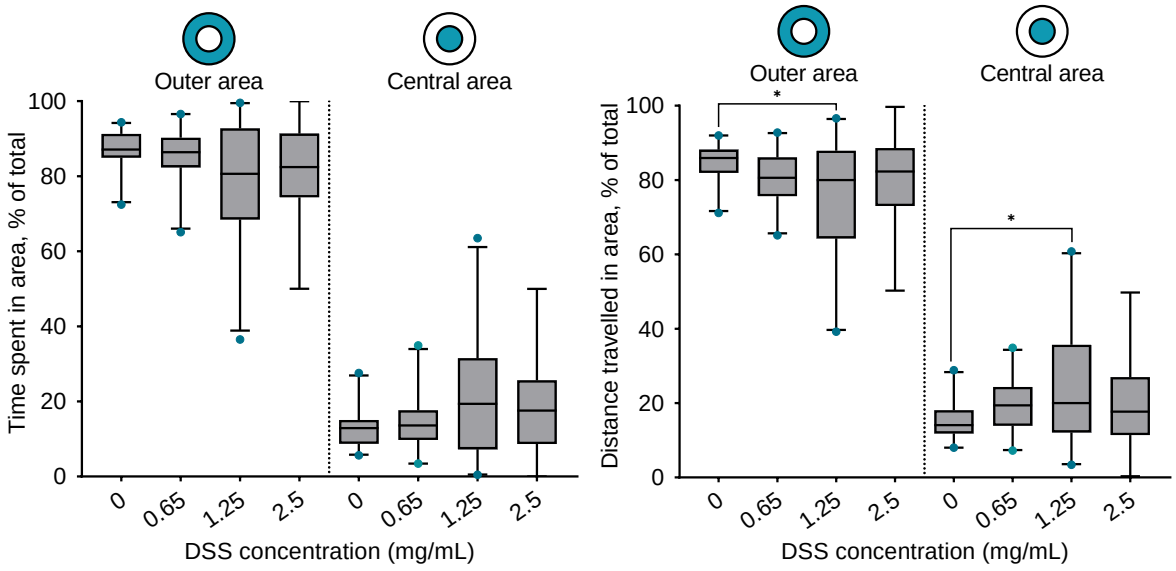
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Distance travelled at different speed thresholds: fast/bursting (>5mm/s), normal/cruising (1 to 5 mm/s), and inactive/drifting (<1 mm/s)

Figure 2.34 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=21 larvae per group, with the exception of 2.5mg/mL, where n=9 due to increased mortality rates. Exposure to 1.25 and 2.5 mg/mL DSS significantly lowers total distance travelled (a). This decrease was also significant in the average speed (b) exposed to 2.5 mg/mL DSS *vs* controls. At these concentrations, significantly less of the movement is performed at bursting speeds, with higher distances travelled while drifting (c).



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well as a percent of total distance travelled

Figure 2.35 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 68 hpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central areas of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=21 larvae per group, with the exception of 2.5mg/mL, where n=9 due to increased mortality rates. While larvae exposed to 1.25 and 2.5 mg/mL show a slightly increased preference for spending time in the central area, this difference was not significant. Larvae exposed to 1.25 mg/mL DSS show a significant preference for moving in the central area of the well.

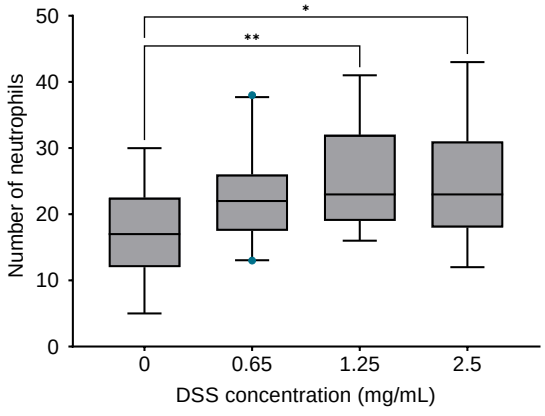


Figure 2.36 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (as demarcated in Fig. 2.4) in Tg(mpx:GFP)i114 larval zebrafish after 48h of exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 68 hpf. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=17 (Control), 20 (0.65 mg/mL), 19 (1.25 mg/mL) and 15 (2.5 mg/mL) larvae per group. Exposure to 1.25 and 2.5 mg/mL DSS significantly increases the number of neutrophils in the gut *vs.* controls.

2.4.4 Phase Three: Behaviour and immune profile of zebrafish larvae exposed to Dextran Sulfate Sodium (DSS) for 10 days

Following phase two experiments, as determined in the pre-established methodological approach, phase three will consist of prolonged exposures to DSS, from 3 to 13 dpf.

2.4.4.1 Pilot studies

A 10-day pilot exposure with DSS was conducted using the non-lethal concentrations determined in the previous 48-hour experiments: 0.325, 0.65 and 1.3 mg/mL. At 72 hours postfertilization, after hatching, 13 larvae were loaded into each well of a 6-well plate containing the selected concentrations of DSS in recirculating system water, along with a water-only control. A total of 52 larvae were used, with 13 larvae per exposure group. Zebrafish larvae were maintained as described in Section 2.3. However, mortality rates above control baseline levels were observed in larvae exposed to 0.65 and 1.3 mg/mL, and this experiment was promptly terminated by MS-222 overdose at 9 dpf (Fig. 2.37a).

To avoid any unexpected mortality, a new pilot 10-day exposure was set up with concentrations 3 times lower than the previous experiment (0.001, 0.0033, 0.01, 0.033, 0.1 mg/mL) (Fig. 2.37b). A low mortality rate up to 30% in all groups after external feeding starts is considered natural and acceptable and is generally observed in control groups. This is due to some larvae failing to initiate external feeding after depletion of the yolk sac.

The range of concentrations used in the second experiment disrupted the behaviour of larvae when compared to the control. While there was a noticeable disruption in response to dark and light stimuli (Fig. 2.38), an unexpected increase in distance travelled and speed was also observed, coupled with an increase in movements at cruising speeds and a decrease in bursting movements. However, these changes are not significant and display high variability due to the low sample size (n=4).

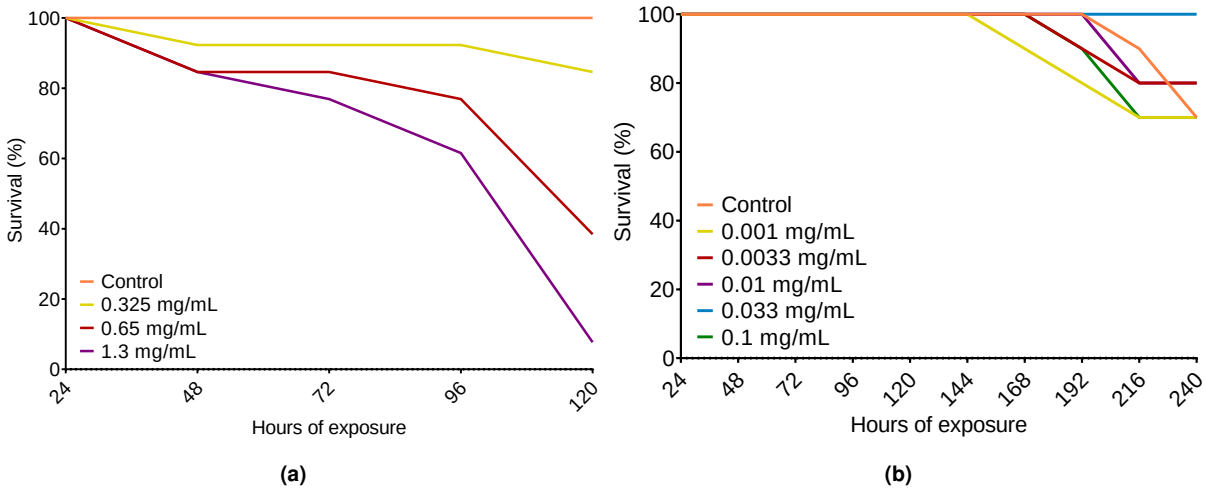


Figure 2.37 – Mortality of zebrafish after 10-day exposure to DSS at different concentrations. Larvae were introduced to the dilutions from 3dpf. (a) N=13 larvae per group. This experiment was terminated at 9 dpf due to elevated mortality rates. (b) N=10 larvae per group. Exposure to DSS concentrations ranging from 0.001 to 0.1 mg/mL resulted in mortality rates under 30% for all exposure groups. A low mortality rate in all groups after external feeding starts is considered natural and acceptable.

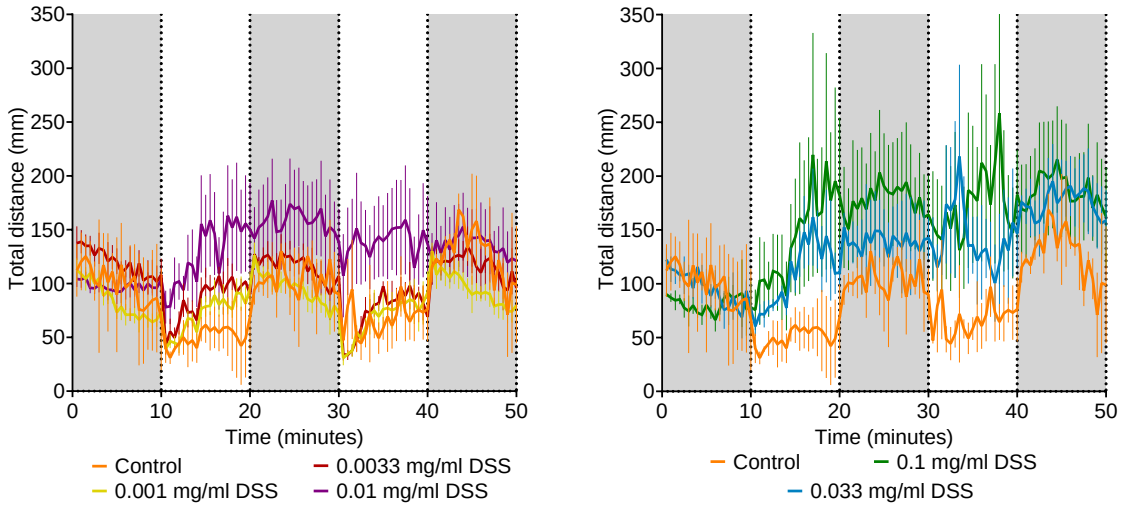
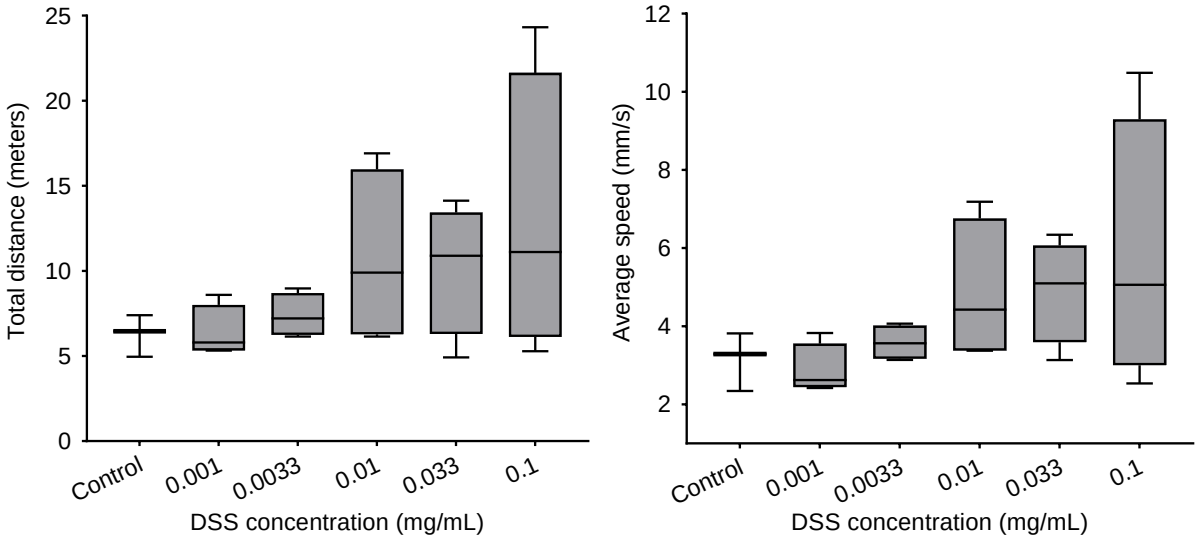


Figure 2.38 – Effect of alternating light–dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Free swimming hatched larvae were introduced to the dilutions from 3dpf. An initial 10-minute period of darkness was followed by four alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=4 larvae per group. Exposure to DSS ranging from 0.001 to 0.1 mg/mL disrupts the larvae response to dark and light stimuli, coupled with an increase in overall movement.

Table 2.14 – Number of time points with statistically significant difference compared to control for data in Fig. 2.38. Data represent the response to alternating light-dark periods on locomotion after 10-day of exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). N=4 larvae per group.

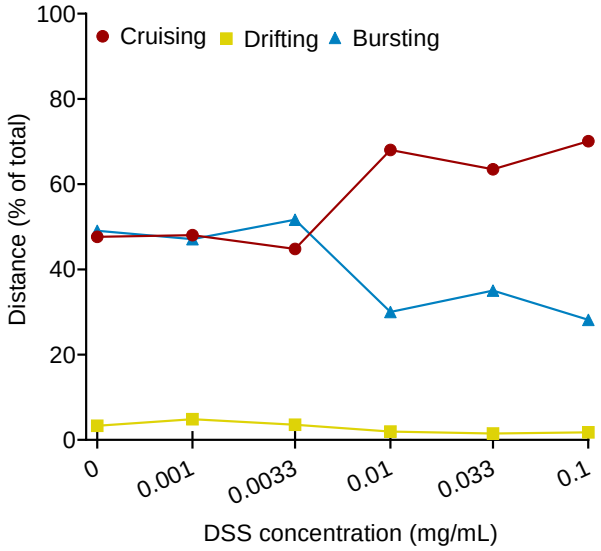
	Control vs. 0.001 mg/ml	Control vs. 0.0033 mg/ml	Control vs. 0.01 mg/ml	Control vs. 0.033 mg/ml	Control vs. 0.1 mg/ml
Time points with statistically significant difference *vs.* control	0	0	0	1	9

The number of neutrophils present in the gut of larvae exposed to DSS increased compared to control, while mortality levels were within the normal range for the length of this experiment. While these results are not statistically significant, this can likely be explained by the smaller sample size (4 larvae per group). As the number of neutrophils present in the gut of larvae exposed to DSS seems to increase compared to control, coupled with the behaviour profile obtained, the results f this pilot warrants further exploration with a larger sample number.



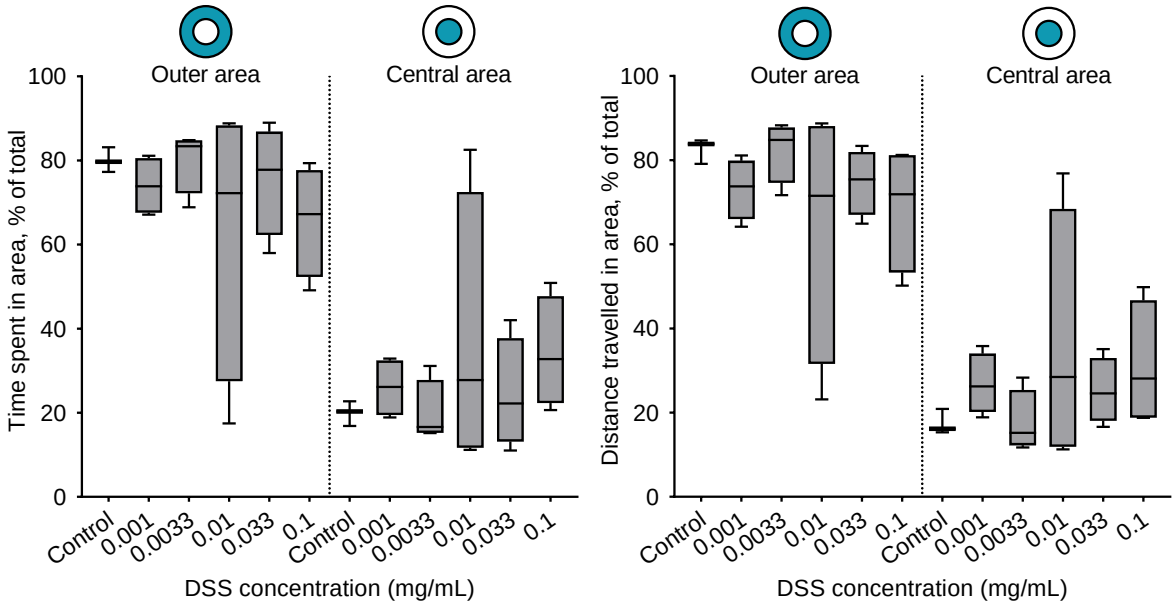
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Distance travelled at different speed thresholds

Figure 2.39 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements $>$5mm/s are considered bursting, and $<$1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=4 larvae per group. Total distance travelled (a) and average speed (b) seem to dose dependently increase in larvae exposed to DSS, with a higher percentage of distance travelled at cruising speeds (c). However, these results are not statistically significant, likely due to the smaller sample size.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well

Figure 2.40 – Assessment of behavioural differences in wild-type larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=4 larvae per group. Thigmotaxis varies between groups with no discernible trend, due to high individual variability and low sample numbers.

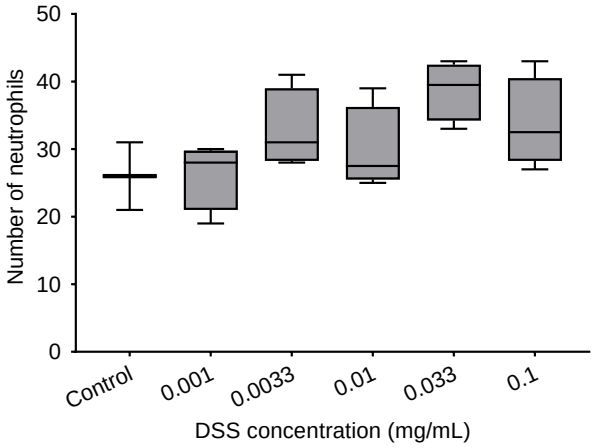


Figure 2.41 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (area as seen demarcated in Fig. 2.4) in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3 dpf. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=4 larvae per group. Exposure to DSS seems to dose-dependently increase the number of neutrophils in the gut. However, these results are not statistically significant, likely due to the smaller sample size.

2.4.4.2 Behaviour and neutrophil infiltration on the gut of Tg(mpx:GFP)i114 zebrafish

Following the pilot study carried out with wild-type AB zebrafish, Tg(mpx:GFP)i114 zebrafish were exposed for 10 days to the DSS concentrations determined in the previous pilot experiment. The study was carried out in 3 independent experiments. In the first two experiments, 21 zebrafish larvae were exposed to 0.001, 0.0033, 0.01, 0.033, and 0.1 mg/mL of DSS and a water-only control (42 larvae per exposure group, in total 252 larvae). An additional exposure was conducted with 0.1 mg/mL (24 larvae), 0.33 mg/mL (48 larvae) and a water-only control (24 larvae). At hours post-fertilization, after hatching, 10 larvae were loaded into each well of 6-well plates containing the selected concentrations of DSS in recirculating system water, along with a water-only control. Zebrafish larvae were maintained as described in the methods section.

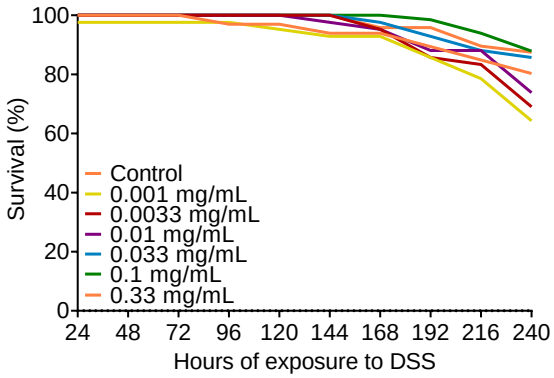


Figure 2.42 – Survival of zebrafish after 10-day exposure to DSS at different concentrations. Larvae were introduced to the dilutions from 3dpf. N=42 (0.001, 0.0033, 0.01, 0.033 mg/mL), 48 (0.33 mg/mL) and 64 (control and 0.33 mg/mL) larvae per group. Exposure to DSS concentrations ranging from 0.001 to 0.1 mg/mL resulted in mortality rates under 30% for all exposure groups, except 0.001 and 0.0033 mg/mL, which displayed mortality rates of 36% and 31%, respectively.

While in the pilot experiment higher concentrations of DSS clearly affected dark-light transition response, the same was not observed at the selected lower concentrations, with dark-light transition response not displaying a clear dose dependent response (Fig. 2.43, Table 2.15). While a higher fraction of time points were significantly different from controls at lower concentrations than at higher ones, these still represent a small proportion of the overall number of timepoints in the 50 minutes analysed (one every 30 seconds, totaling 100). As seen in the pilot, 0.1 mg/mL DSS seems

to increase the distance travelled and speed slightly, but this change was not significant (p=>0.9999)). However, this increase wasn't observed in larvae exposed to 0.33 mg/mL. Larvae exposed to 0.33 mg/mL DSS significantly prefer to spend time in the outer area of the well *vs* the central area, swimming significantly less in the centre of the well. This alteration in thigmotaxis was not coupled with a decrease in total distance *vs* control, pointing to a modulation in anxious behaviour that is not caused by the compound's toxicity. Neutrophil infiltration in the gut was significantly higher in larvae exposed to concentrations equal to or higher than 0.033 mg/mL. As defined in the approach set at the start of this project, these results will further be confirmed through a 10-day exposure using Tg(Mpeg MCherry CAAX)Sh378 zebrafish larvae.

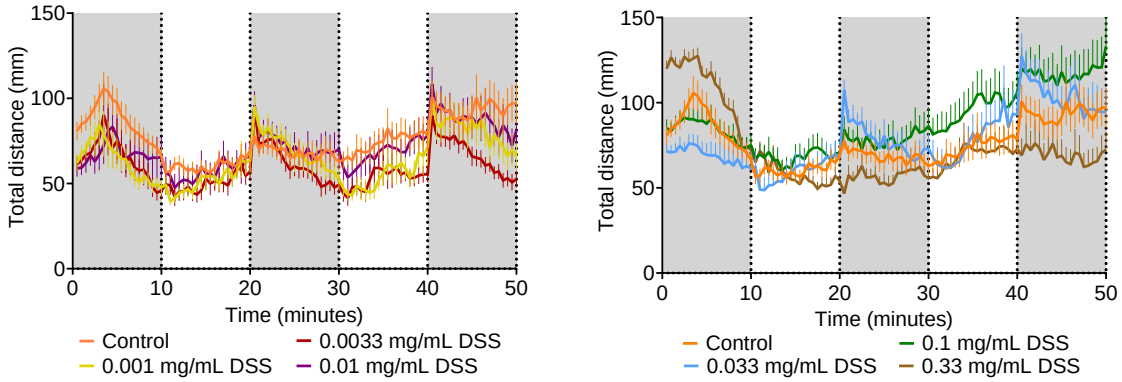
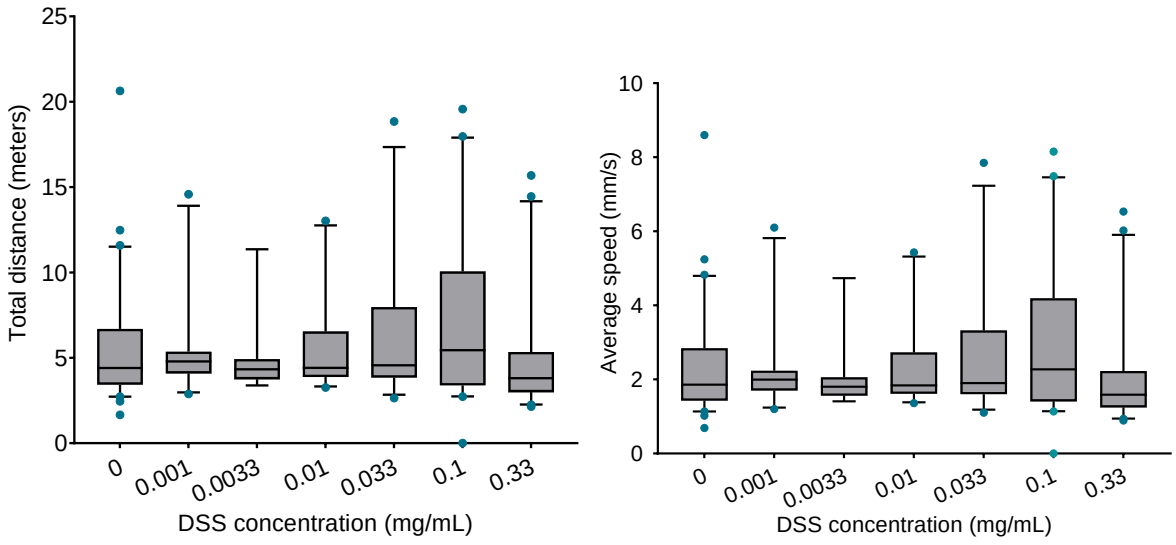


Figure 2.43 – Effect of alternating light–dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Free swimming hatched larvae were introduced to the dilutions from 3 dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=(control), 22 (0.001 mg/mL), 19 (0.0033 mg/mL), 23 (0.01 mg/mL), 24 (0.033 mg/mL), 41 (0.1 mg/mL) and 42 (0.33 mg/mL) larvae per group. Exposure to 0.001 to 0.1 mg/mL DSS had no effect on the response of larvae to light and dark.

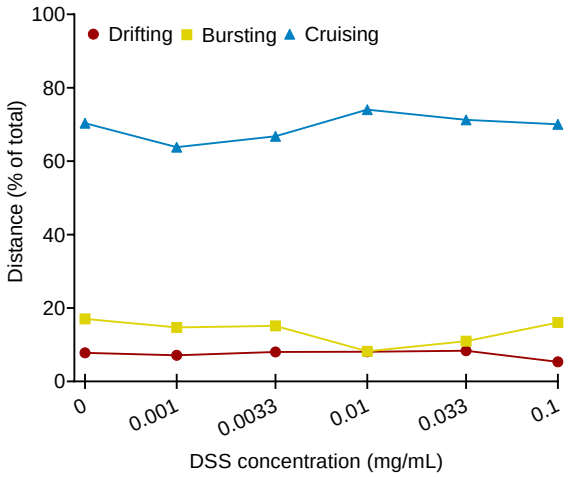
Table 2.15 – Number of time points with statistically significant difference compared to control for data in Fig. 2.43. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). N=(control), 22 (0.001 mg/mL), 19 (0.0033 mg/mL), 23 (0.01 mg/mL), 24 (0.033 mg/mL), 41 (0.1 mg/mL) and 42 (0.33 mg/mL) larvae per group.

	Control vs. 0.001 mg/ml	Control vs. 0.0033 mg/ml	Control vs. 0.01 mg/ml	Control vs. 0.033 mg/ml	Control vs. 0.1 mg/ml	Control vs. 0.33 mg/mL
Time points with statistically significant difference *vs.* control	15	18	6	1	0	7



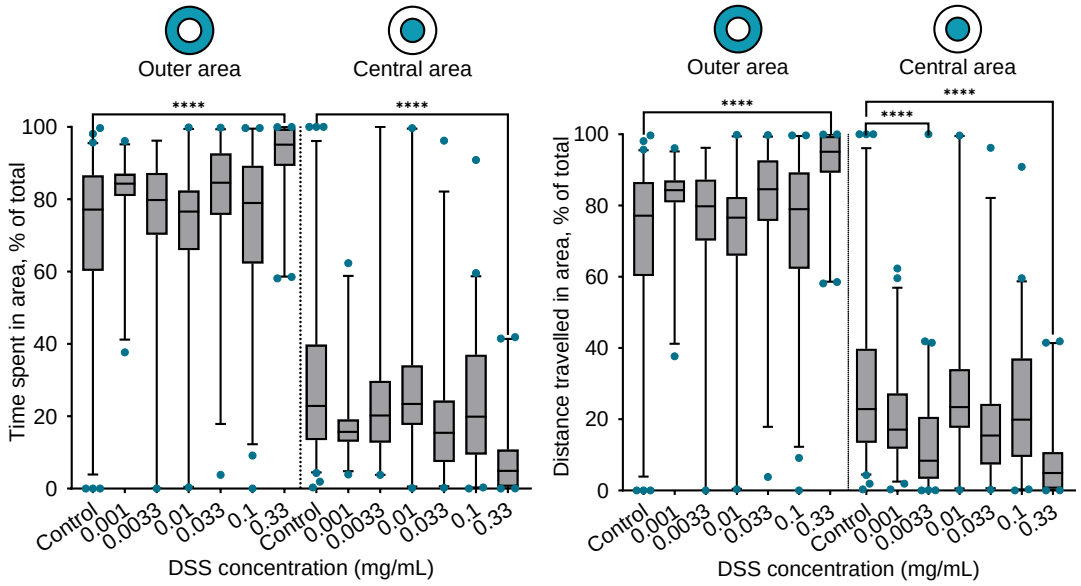
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Distance travelled at different speed thresholds: fast/bursting (>5mm/s), normal/cruising (1 to 5 mm/s), and inactive/drifting (<1 mm/s)

Figure 2.44 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=(control), 22 (0.001 mg/mL), 19 (0.0033 mg/mL), 23 (0.01 mg/mL), 24 (0.033 mg/mL), 41 (0.1 mg/mL) and 42 (0.33 mg/mL) larvae per group. 0.1 mg/mL DSS slightly increases distance travelled and speed, but this change was not significant. This increase was not seen in larvae exposed to 0.33 mg/mL.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well

Figure 2.45 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=72 (control), 22 (0.001 mg/mL), 19 (0.0033 mg/mL), 23 (0.01 mg/mL), 24 (0.033 mg/mL), 41 (0.1 mg/mL) and 42 (0.33 mg/mL) larvae per group. Thigmotaxis was significantly affected in larvae exposed to 0.33 mg/mL DSS, with increased preference for the outer area of the well.

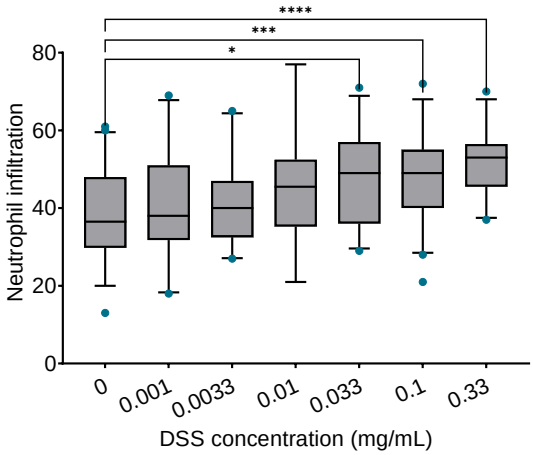


Figure 2.46 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (as demarcated in Fig. 2.4) in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=42 (control), 22 (0.001 mg/mL), 21 (0.0033 mg/mL), 18 (0.01 mg/mL), 25 (0.033 mg/mL), 41 (0.1 mg/mL) and 29 (0.33 mg/mL) larvae per group. Exposure to 0.033, 0.1 and 0.33 mg/mL DSS significantly increases the number of neutrophils in the gut.

2.4.4.3 Behaviour and macrophage infiltration on the gut of Tg(Mpeg MCherry CAAX)Sh378 zebrafish

Tg(Mpeg MCherry CAAX)Sh378 zebrafish were exposed for 10 days to the same DSS concentrations used above, with the exception of 0.33 mg/mL. The study was carried out in 2 independent experiments. A total of 228 larvae were used, with 38 larvae per exposure group. Athours post-fertilization, after hatching, 8 to 10 larvae were loaded into each well of 6-well plates containing the selected concentrations of DSS in recirculating system water, along with a water-only control. Zebrafish larvae were maintained as described in the methods section.

Exposure to DSS from 0.001 to 0.1 mg/mL did not markedly affect dark-light transition response, as also seen in the previous experiment (Fig. 2.48, Table 2.16). However, in this case, overall locomotion appeared to decrease for all exposure groups compared to controls, as seen in Fig. 2.49. DSS up to 0.1 mg/mL did not increase the distance travelled and speed or disrupt thigmotactic behaviour. These results are consistent with those seen Tg(mpx:GFP)i114 larvae, with only concentrations of 0.33 mg/ml significantly affecting thigmotaxis.

Macrophage infiltration in the gut was significantly higher in larvae exposed to 0.1 mg/mL, with a non-significant increase also seen at 0.033 mg/mL. These results are consistent with the inflammatory effect of DSS observed in previous experiments.

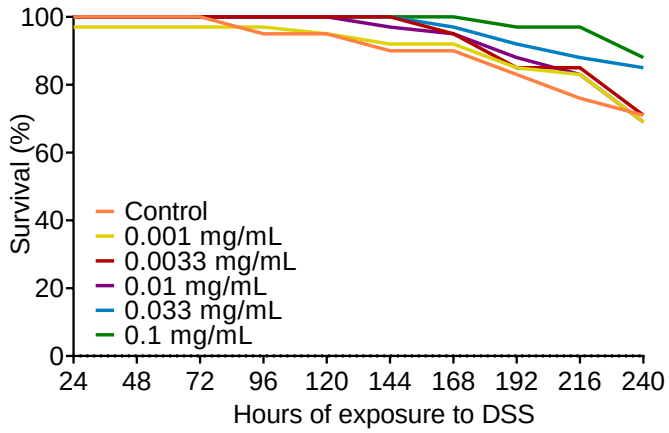


Figure 2.47 – Survival of zebrafish after 10-day exposure to DSS at different concentrations. Larvae were introduced to the dilutions from 3dpf. N=38 larvae per group. Exposure to DSS concentrations ranging from 0.001 to 0.1 mg/mL resulted in mortality rates under 31% for all exposure groups.

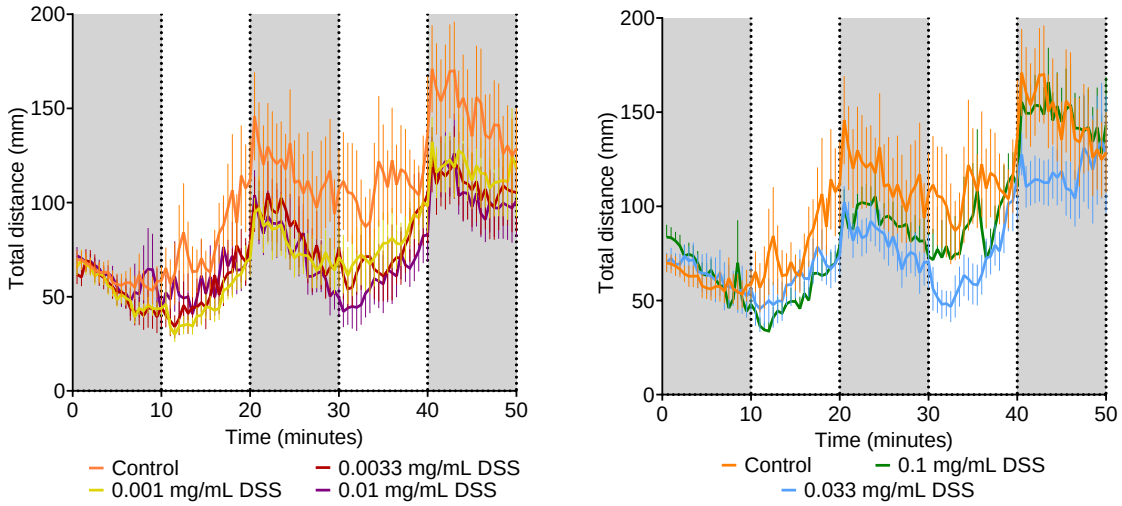
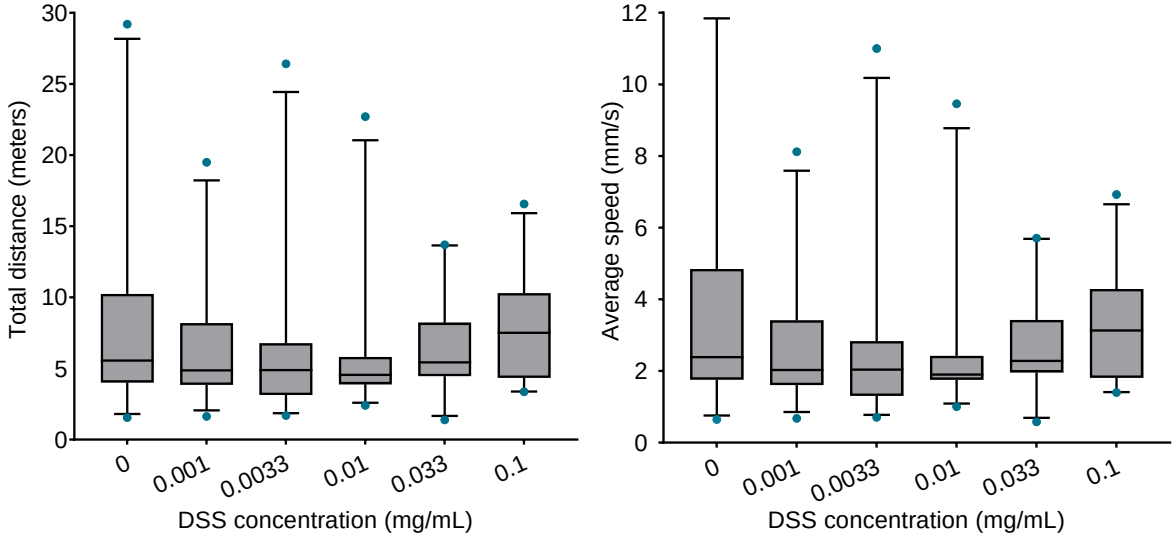


Figure 2.48 – Effect of alternating light–dark periods on locomotion in Tg(Mpeg MCherry CAAX)Sh378 larval zebrafish after 10-day exposure to increasing doses of DSS. Free swimming hatched larvae were introduced to the dilutions from 3 dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=22 to 24 larvae per group. Exposure to DSS ranging from 0.001 to 0.1 mg/mL has no effect the response of larvae to light and dark, but causes a overall decrease in locomotion.

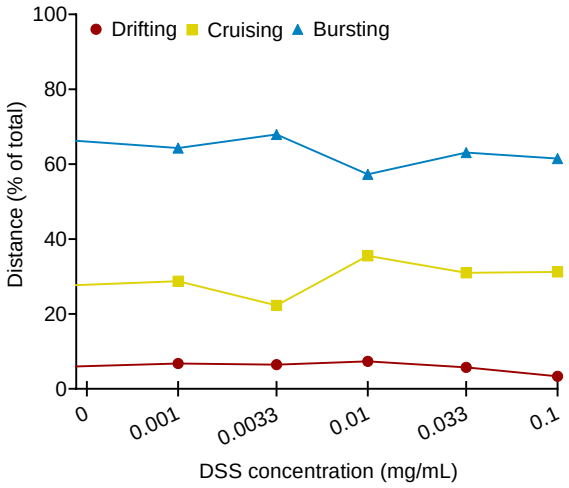
Table 2.16 – **Number of time points with statistically significant difference compared to control for data in Fig. 2.48.** Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). N=22 to 24 larvae per group.

	Control vs. 0.001 mg/ml	Control vs. 0.0033 mg/ml	Control vs. 0.01 mg/ml	Control vs. 0.033 mg/ml	Control vs. 0.1 mg/ml
Time points with statistically significant difference *vs.* control	2	3	12	8	0



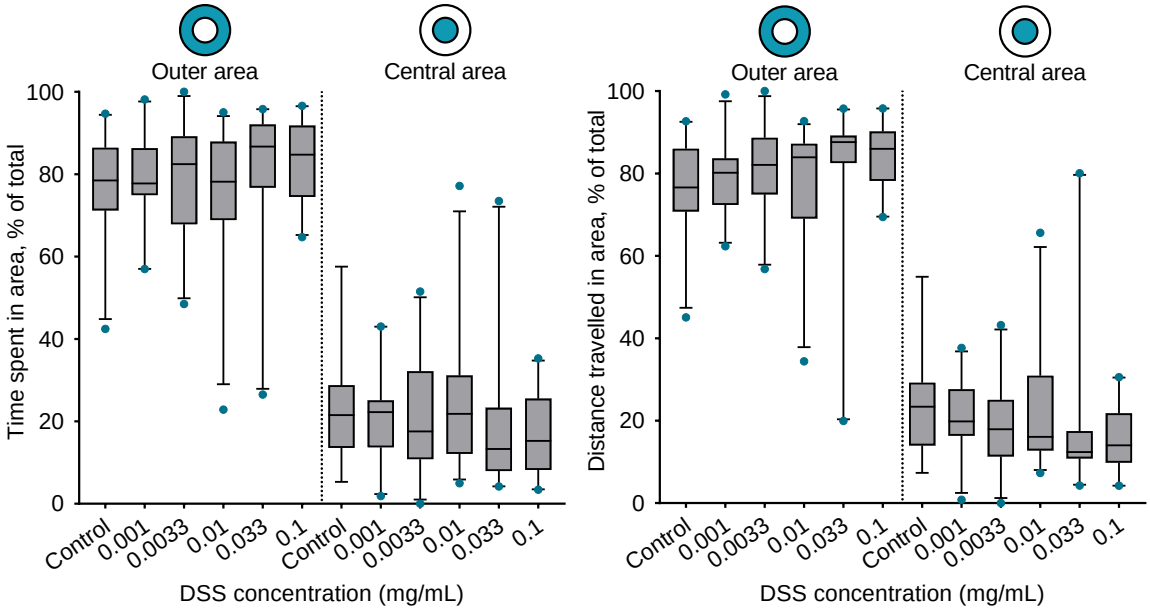
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Distance travelled at different speed thresholds

Figure 2.49 – Assessment of behavioural differences in Tg(Mpeg MCherry CAAX)Sh378 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=22 to 24 larvae per group. Total distance travelled (a) and average speed (b) were not significantly affected by DSS.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well

Figure 2.50 – Assessment of behavioural differences in Tg(Mpeg MCherry CAAX)Sh378 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=23 (control, 0.0033 mg/mL, 0.01 mg/mL), 24 (0.001 mg/mL), and 22 (0.033 and 0.1 mg/mL) larvae per group. Thigmotaxis was not significantly affected by DSS concentrations up to 0.1 mg/mL.

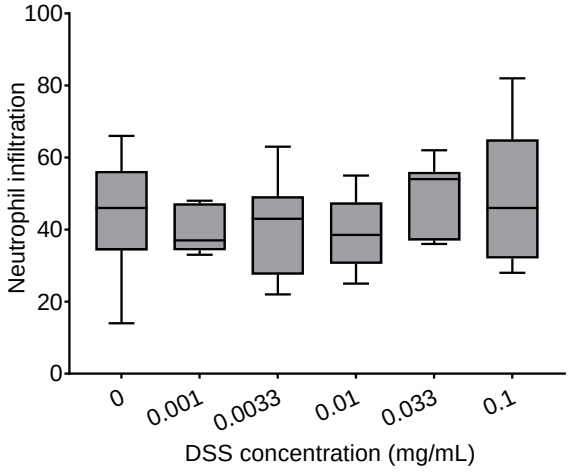


Figure 2.51 – Quantification of infiltrating macrophages in the mid and posterior section of the gut (area as seen demarcated in Fig. 2.4) in Tg(Mpeg MCherry CAAX)Sh378 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=16 (control), 14 (0.001 mg/mL), 19 (0.0033 mg/mL), 20 (0.01 mg/mL), 18 (0.033 mg/mL), 17 (0.1 mg/mL) larvae per group. Exposure to 0.1 mg/mL DSS significantly increased the number of macrophages in the gut.

2.4.4.4 Behaviour of AB wild-type zebrafish

Considering the results obtained thus far with DSS, questions remain concerning the behaviour of zebrafish larvae exposed to 0.1 and 0.33 mg/mL. With this uncertainty in mind, an additional experiment was conducted. Samples were preserved for future transcriptomic studies.

AB wild-type zebrafish were exposed for 10 days to 0.1 and 0.33 mg/mL DSS. A total of 90 larvae were used, with 30 larvae per exposure group. Athpf, after hatching, 10 larvae were loaded into each well of 6-well plates containing the selected concentrations of DSS in recirculating system water, along with a water-only control. Zebrafish larvae were maintained as described in the methods section.

In this experiment, 0.33 mg/mL DSS caused unexpected mortality levels in larvae not previously seen, with only 53% of larvae surviving after 10 days of exposure (*vs.* 75% observed in the control group). This reduced survival compared to the previously established emerged only on the last day of the experiment, when animals were promptly terminated through MS-222 overdose.

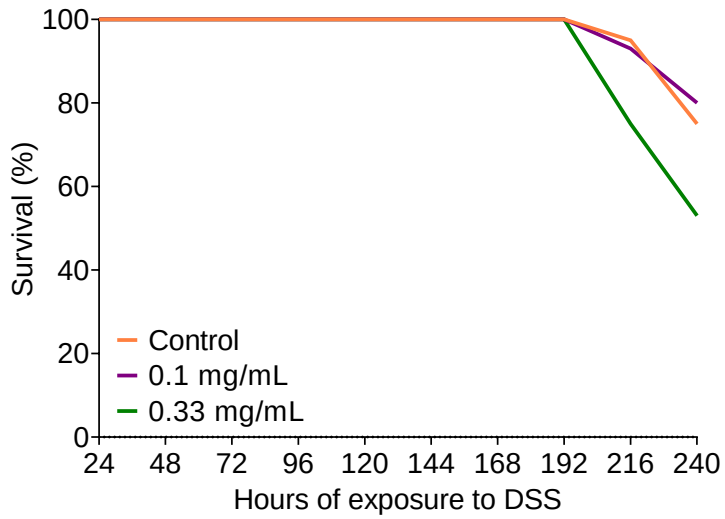


Figure 2.52 – Survival of AB WT zebrafish after 10-day exposure to DSS at different concentrations. Larvae were introduced to the dilutions from 3 dpf. N=30 larvae per group. Exposure to 0.33 mg/mL DSS resulted in mortality rates of 47%.

As previously observed, exposure to DSS did not have a significant effect on the response of larvae to light and dark stimuli. The peak in movement observed in the control group in the first 10 minutes was limited to one plate. While the DSS seems to

have an effect in the movement levels of larvae (Fig. 2.53) this was change was not significant at a time point basis (Table 2.17). Equally and consistently with previous experiments, 0.33 mg/mL DSS caused an increase in distance travelled and average speed. However, these changes were also not statistically significant (p=0.135 and p=0.1853, respectively). Thigmotaxis was affected in larvae exposed to 0.1 and 0.33 mg/mL DSS, with an increased preference for the outer *vs* the central area compared to controls. This preference was also clearly seen in GFP larvae exposed to 0.33 mg/mL DSS.

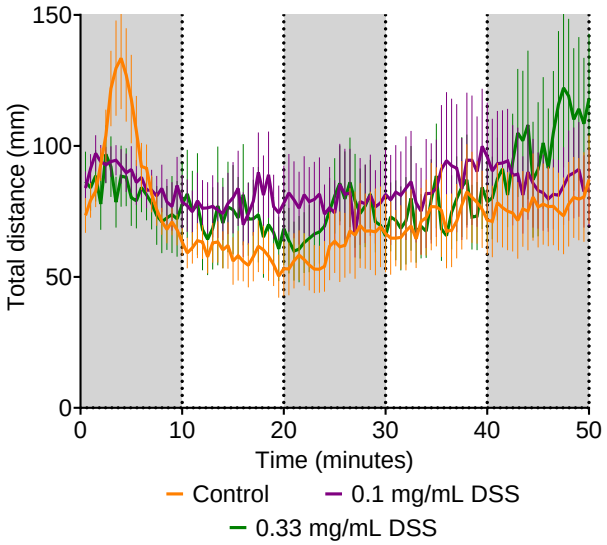
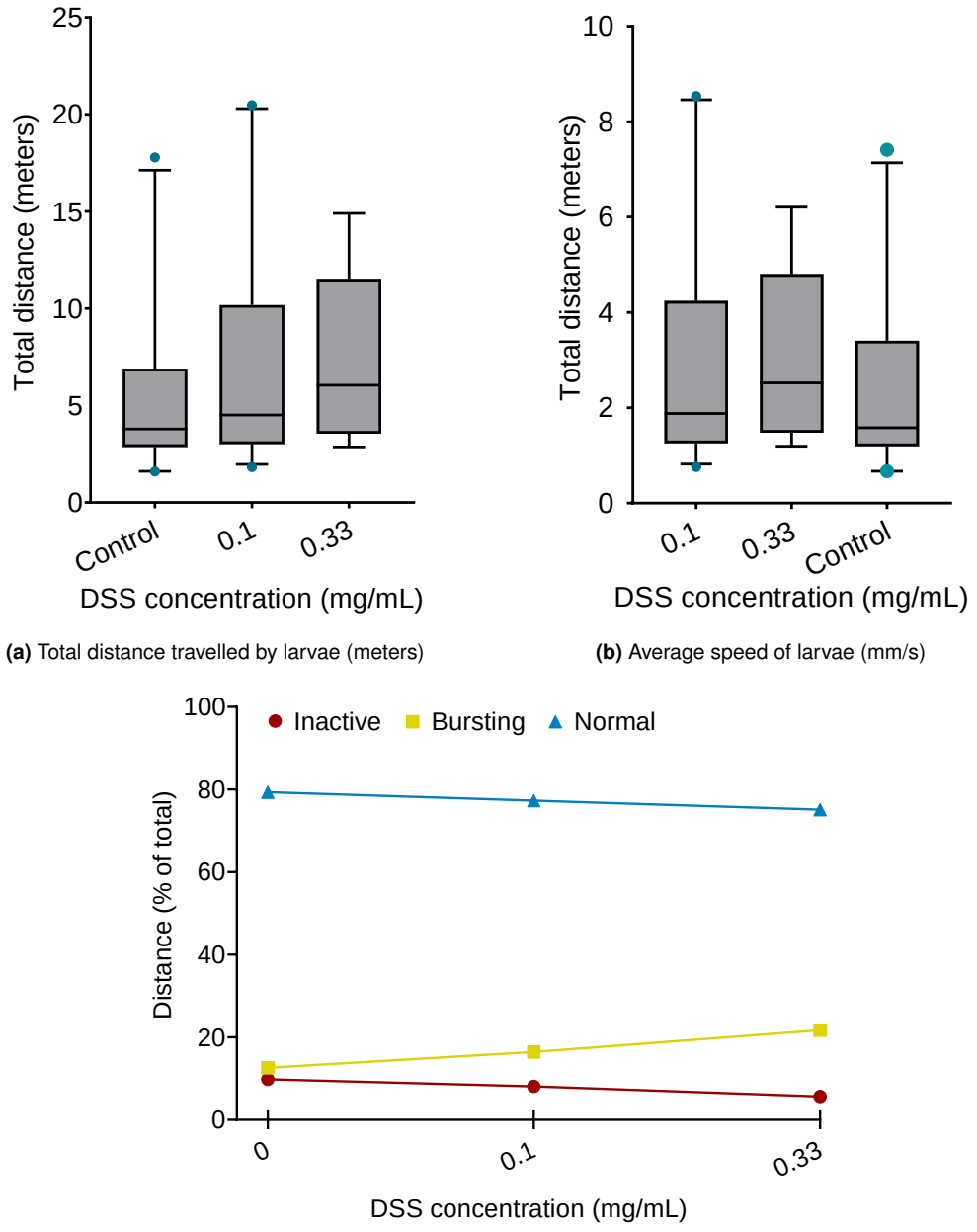


Figure 2.53 – Effect of alternating light–dark periods on locomotion in wild-type larval zebrafish after 10-day exposure to increasing doses of DSS. Free swimming hatched larvae were introduced to the dilutions from 3 dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=22 (control), 21 (0.1 mg/mL) and 17 (0.33 mg/mL) larvae per group. Exposure to 0.1 and 0.33 mg/mL DSS has no effect the response of larvae to light and dark.

Table 2.17 – Number of time points with statistically significant difference compared to control for data in Fig. 2.53. Data represent the response to alternating light-dark periods on locomotion after 10-day exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). N=22 to 24 larvae per group.

	Control vs. 0.1 mg/ml	Control vs. 0.33 mg/ml
Time points with statistically significant difference *vs.* control	0	2

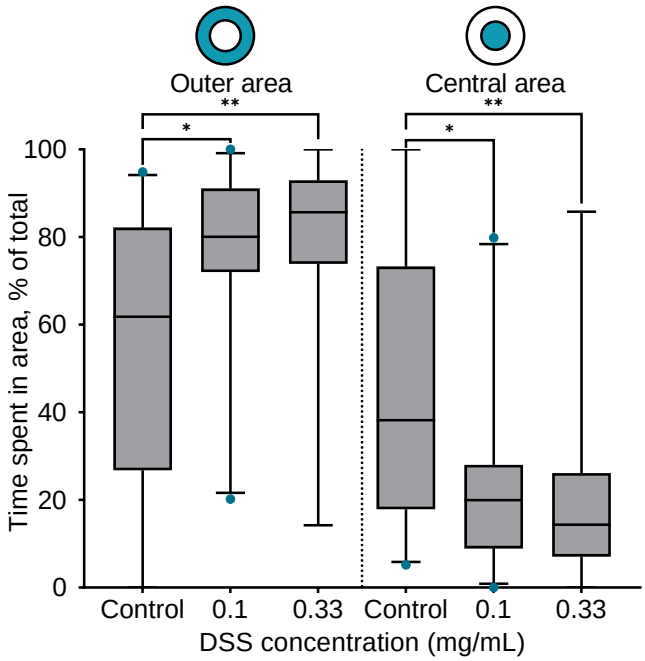


(a) Total distance travelled by larvae (meters)

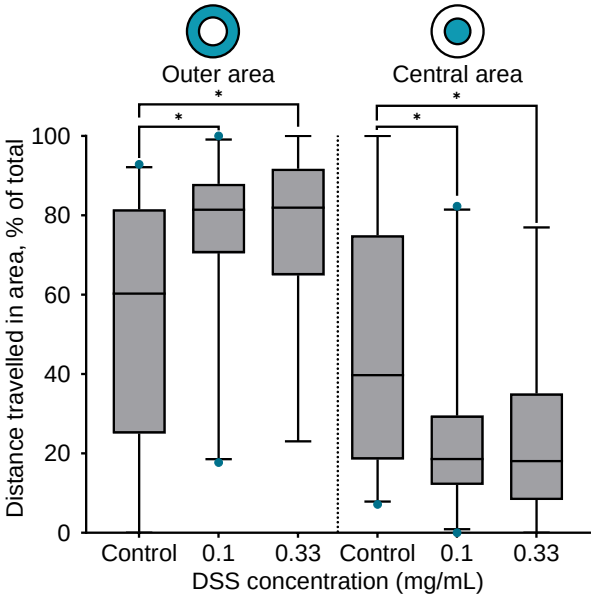
(b) Average speed of larvae (mm/s)

(c) Distance travelled at different speed thresholds

Figure 2.54 – Assessment of behavioural differences in wild-type larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements $>$5mm/s are considered bursting, and $<$1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=22 (control), 21 (0.1 mg/mL) and 17 (0.33 mg/mL) larvae per group. Exposure to 0.33 mg/mL DSS increased total distance travelled (a) and average speed (b). However, these changes were not statistically significant (p=0.135 and p=0.1853, respectively).



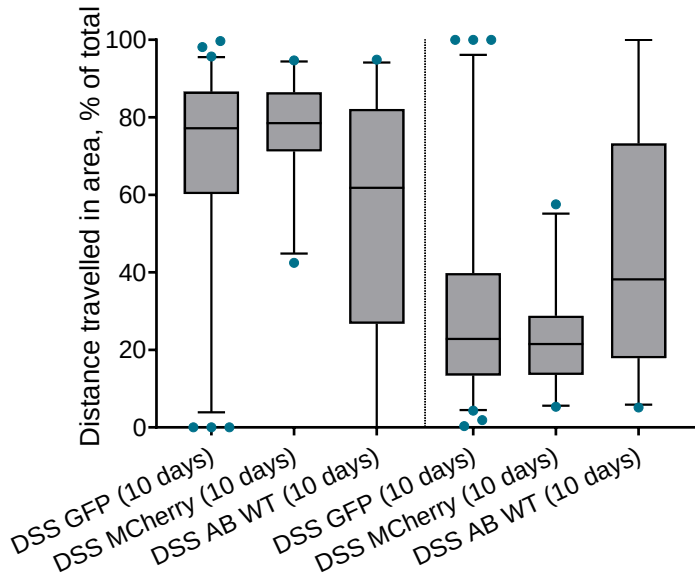
(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time



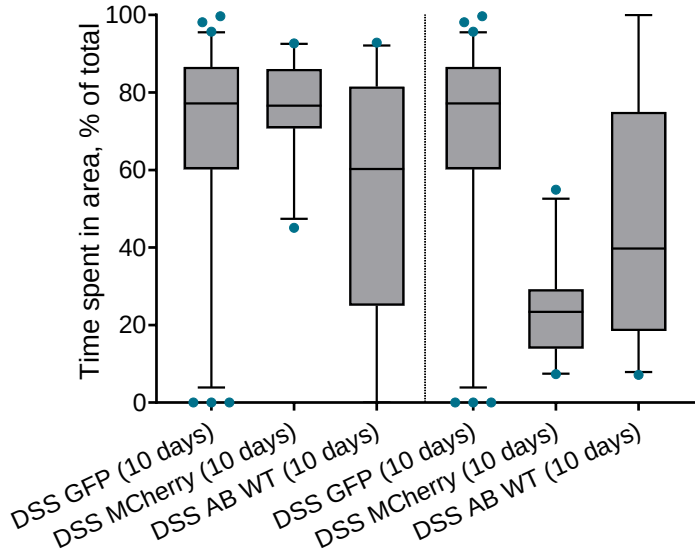
(b) Distance travelled by larvae in the central and outer area of the well

Figure 2.55 – Assessment of behavioural differences in wild-type larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=22 (control), 21 (0.1 mg/mL) and 17 (0.33 mg/mL) larvae per group. Thigmotaxis was significantly affected by DSS, with larvae exposed to 0.1 and 0.33 mg/mL DSS, showing increased preference for the outer *vs* the central area when compared to controls.

Despite appearing lower in the AB WT larvae exposure, thigmotaxis did not significantly vary among control groups in the 10-day exposure DSS experiments (Fig. 2.56).



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time



(b) Distance travelled by larvae in the central and outer area of the well

Figure 2.56 – Comparison of behavioural differences in control larval zebrafish across 3 distinct experiments. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Thigmotaxis did not significantly vary in controls across these experiments.

2.4.5 Zebrafish high fat and high cholesterol diet

Many studies performed in rodents have shown that overfeeding and a high-fat diet (HFD) can alter the intestinal microbiota and perturb immune homeostasis, inducing intestinal inflammation. These alterations lead to the activation of innate immunity-mediated low-grade inflammation, likely to be chronically activated and modulated by pro-inflammatory cytokines (Devkota et al., 2012; Arias-Jayo et al., 2018). Recently, the zebrafish has emerged as an alternative vertebrate model for the study of lipid metabolism and metabolic diseases (Faillaci et al., 2018; Löhr and Hammerschmidt, 2011; Seth et al., 2013), with studies showing that diet-induced obesity in zebrafish shares common pathophysiological pathways with obesity in mammals (Hasumura et al., 2012; Leibold and Hammerschmidt, 2015; Oka et al., 2010; Tainaka et al., 2011). Furthermore, it has been demonstrated that feeding zebrafish high-fat or high-cholesterol diets changes the intestinal microbial community composition and induces intestinal inflammation (Landgraf et al., 2017; Arias-Jayo et al., 2018; Progatzky et al., 2014; Faillaci et al., 2018), as seen in Table 2.18.

Table 2.18 – Zebrafish high-fat and high cholesterol diets known to induce intestinal inflammation

Reference	Diet	Zebrafish age	Exposure duration
Progatzky et al., 2014	Clotted cream (63.5 g of fat, 170 mg of cholesterol, 2.2 g of carbohydrate and 1.6 g of protein per 100 g) at a 1:10 dilution in system water	Larvae (6 dpf)	6h
	4-8% (w/w) cholesterol in food	Adult	2 times/day, 10 days
Landgraf et al. (2017)	Overfed 60 mg peeled Artemia salina cysts (22% fat, 44% proteins, 16% carbohydrates)	Adult	1 time/day, 8 weeks
	Combination of artemia (5 mg artemia) and 30 mg egg yolk powder (59% fat, 32% proteins, 2% carbohydrates)	Adult	1 time/day, 8 weeks
Arias-Jayo et al. (2018)	10% (w/w) of cocoa butter in food	Larvae (5 dpf)	3 times/day, 30 days

A high-fat diet with 4% added cholesterol was custom ordered from SPAROS (Olhão, Portugal). Detailed composition is presented in Section 2.3.3, Tables 2.1 and 2.2. The protocol for comparison of the effect of high-fat versus normal diets in zebrafish larvae was the same as the 10-day exposure above mentioned, extended to 15 dpf. At 5 dpf, 10 Tg(mpx:GFP)i114 zebrafish larvae were loaded into each well of

6-well plates. Starting at 5 dpf, larvae were fed twice per day for 10 days with regular granular food <100um (SPAROS, Olhão, Portugal) or with the custom-made high-fat diet. A total of 180 larvae were used, with 60 larvae per exposure group. Zebrafish larvae were maintained as described in Section 2.3.

Overall, larvae fed with regular diet or HFD with 4% cholesterol showed the same response to dark/light stimuli (Fig. 2.57, with no significant differences between time points for larvae fed HFD versus regular diet. Although there was a slight decrease in distance travelled and speed of larvae fed HFD, this difference was not statistically significant (p=0.0904 and p=0.1772, respectively). There was a small statistically significant decrease (p=0.0364) in movements done at cruising speeds coupled with a non-significant increase in drifting (p=0.0522) for larvae fed a high-fat diet. Larvae fed the two different diets show no change in thigmotactic behaviours. While there was a slight decrease in neutrophil infiltration in the gut of larvae fed HFD, this difference was not statistically significant (p=0.0997).

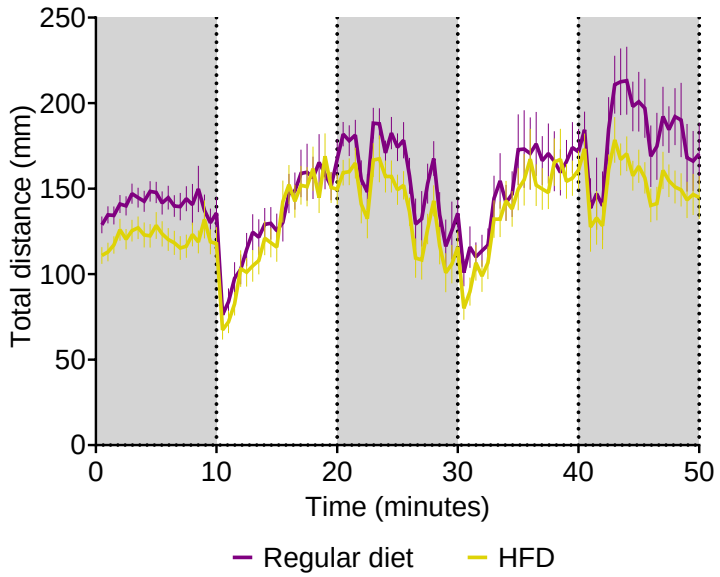
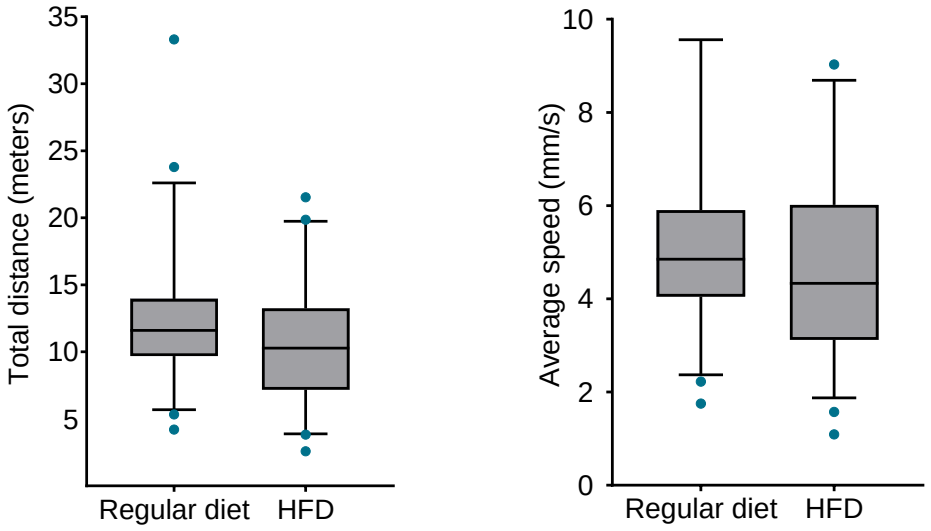
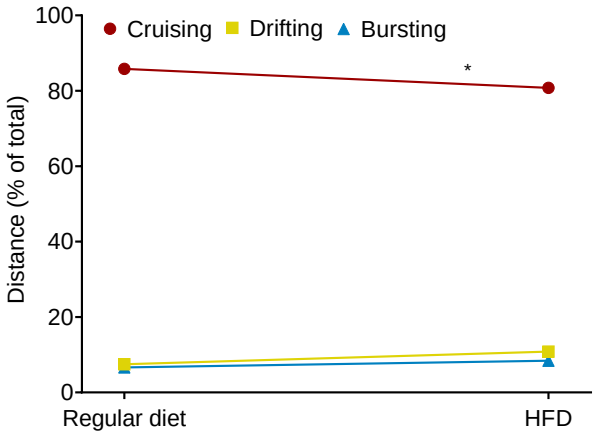


Figure 2.57 – Effect of alternating light–dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 10 days feeding with regular zebrafish feed or high-fat, high-cholesterol diet. Feeding started at 5dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=47 (regular diet) and 48 (HFD) larvae per group. Feeding zebrafish larvae with HFD for 10 days did not affect dark-light transition response when compared to larvae fed with a regular diet.



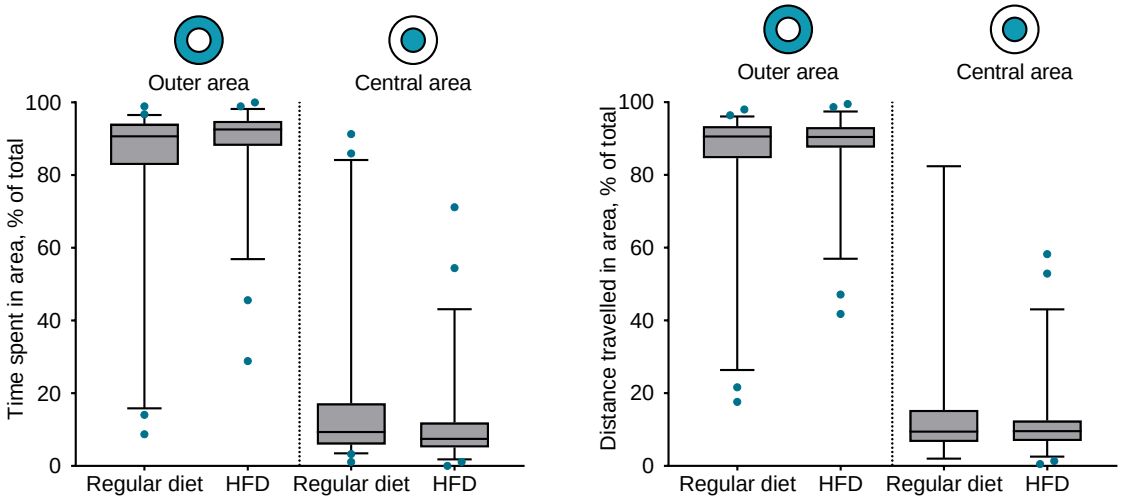
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)



(c) Distance travelled at different speed thresholds: fast/bursting (>5mm/s), normal/cruising (1 to 5 mm/s), and inactive/drifting (<1 mm/s)

Figure 2.58 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 10 days feeding with regular zebrafish feed or high-fat, high-cholesterol diet. Feeding started at 5dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=47 (regular diet) and 48 (HFD) larvae per group. Feeding zebrafish larvae with HFD for 10 days did not affect total distance travelled (a) and average speed (b) when compared to larvae fed with a regular diet. Larvae fed with HFD showed a small statistically significant decrease in preference for moving at cruising speeds, couples with a non significant increase in drifting.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well

Figure 2.59 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 10 days feeding with regular zebrafish feed or high-fat, high-cholesterol diet. Feeding started at 5dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=47 (regular diet) and 48 (HFD) larvae per group. Feeding zebrafish larvae with HFD for 10 days did not affect thigmotaxis when compared to larvae fed with a regular diet.

While it has been previously shown that a single short feed of high cholesterol diet can induce a significant increase of neutrophils in the gut of zebrafish larvae (Progatzky et al., 2014), we were not able to replicate these results. Although different from the expected, these results are not wholly unexpected. During this experiment, several difficulties quickly became evident. Firstly, the dosage of this food was difficult since, due to the high-fat content, the small granules were sticky and clumped together. This resulted in inconsistent feeding of larvae, with no guarantee that the intake of fat and cholesterol was similar for all animals. Furthermore, the HFD quickly deteriorated the water quality compared to the regular diet.

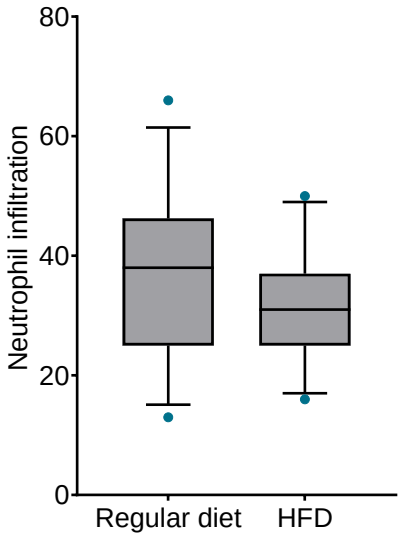


Figure 2.60 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (as demarcated in Fig. 2.4) in Tg(mpx:GFP)i114 larval zebrafish after 10 days feeding with regular zebrafish feed or high-fat, high-cholesterol diet. Feeding started at 5dpf. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=26 (regular diet) and 23 (HFD) larvae per group. Feeding zebrafish larvae with HFD for 10 days did not significantly affect neutrophil infiltration in the gut when compared to larvae fed with a regular diet.

2.4.6 Recovery of behavioural and immune effects of DSS using anti-inflammatory drug dexamethasone

Dexamethasone is a glucocorticoid and an anti-inflammatory drug that has been widely used in zebrafish to counter the effects of chemical and physical pro-inflammatory stimuli by inhibiting macrophage and neutrophil recruitment to the injury site and improving survival (Yang et al., 2014; Zhang et al., 2008). While concentrations of 25 to 100 µg/ml of dexamethasone at 5 dpf for 3 days have been reported to cause ablation of T-cells in surviving zebrafish larvae (M. et al., 2004), 5-day exposure to 100mg/mL dexamethasone has been shown to cause 70% mortality in 5-day post-hatching larvae (Batista et al., 2014). It has also been previously shown that 5 dpf larvae exposed to 50 mg/L DEX or 4dpf display mortality rates of approximately 58% (Wang et al., 2018) and that larvae treated with 10mg/L dexamethasone from approximately 36 hpf to 180 hpf did not significantly display increased mortality compared controls (Voelz et al., 2015). There are other reports of exposure of zebrafish to DEX from 3.9 mg/L to 10mg/L for which no mortality was reported in the literature (Ryu et al., 2021; Yin et al., 2019;

Saettele et al., 2022; Mendonça-Gomes et al., 2021).

Due to dexamethasone being insoluble in water and to avoid using solvents, DSP was used. Based on their molecular weights, the 1gr of dexamethasone was considered to be equivalent to 1.316 gr of DSP.

A 10-day pilot exposure with DSP was conducted using concentrations determined through a literature review: 0.013, 0.043,0.132, 0.434, 1.316 mg/L (equivalent to 0.01 to 1 mg/L dexamethasone). Athours post-fertilization, after hatching, 13 larvae were loaded into each well of a 6-well plate containing the selected concentrations of DSP in recirculating system water, along with a water-only control. A total of 60 larvae were used, with 10 larvae per exposure group. Zebrafish larvae were maintained as described in Section 2.3. Despite the lack of mortality reported in the literature at 10 mg/L, larvae exposed to 1.316 mg/L displayed increased mortality rates and were terminated by MS222 overdose at 12 dpf. After 10 days exposure to 0.434 mg/L DSP, mortality rates were 40% (Fig. 2.61). Larvae exposed to lower concentrations had mortality rates under 20%, which are comparable to controls and considered natural and acceptable. No morphological changes were observed in any exposure group.

As the concentrations selected aim to minimise the risk of inducing mortality, further experiments exposed zebrafish to concentrations below 0.132 mg/L (90% survival rate).

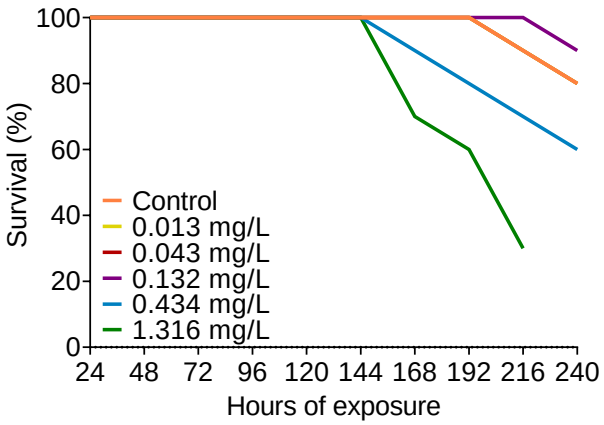


Figure 2.61 – Survival of zebrafish after 10-day exposure to DSP at different concentrations. Larvae were introduced to the dilutions from 3 dpf. N=10 larvae per group. (a) This experiment was terminated at 9 dpf due to elevated mortality rates. (b) Exposure to DSP concentrations ranging from 0.013 to 0.132 mg/L resulted in mortality rates under 20%.

Tg(mpx:GFP)i114 zebrafish were exposed for 10 days to a water control, 0.33 mg/mL DSS, 39.4 µg/L DSP, 132 µg/L DSP, and two co-exposures with 0.33 mg/mL DSS and 39.4 or 132 µg/L DSP. The study was carried out in 2 independent experiments. Athours post fertilisation, after hatching, 10 larvae were loaded into each well of 6-well plates containing the selected concentrations in recirculating system water, along with a water-only control. A total of 252 larvae were used, with 42 larvae per exposure group. Zebrafish larvae were maintained as described in Section 2.3.

While larvae exposed to DSS and DSP alone displayed mortality rates comparable to controls, zebrafish co-exposed with 0.33 mg/mL DSS and either 39.4 and 132 µg/L DSP had mortality rates of 40% (Figure 2.62), indicating a potential higher toxicity caused by the mixture.

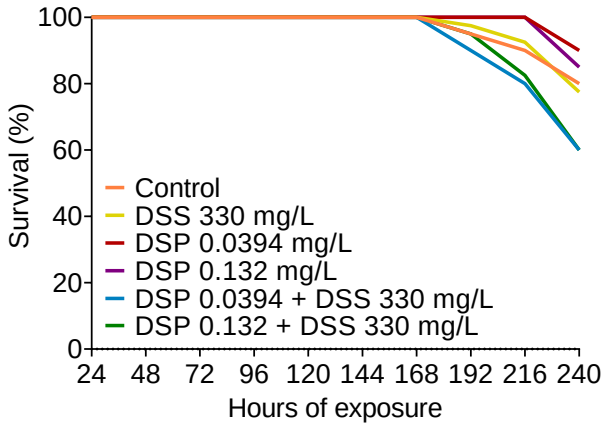


Figure 2.62 – Survival of zebrafish after 10-day single exposure to 0.33 mg/mL DSS, 39.4 µg/L DSP, and 132 µg/L DSP, and two co-exposures with 0.33 mg/mL DSS and 39.4 and 132 µg/L DSP. Larvae were introduced to the dilutions from 3dpf. Larvae exposed to DSS and DSP alone displayed mortality rates comparable to controls, while co-exposure with 0.33 mg/mL DSS and either 39.4 and 132 µg/L DSP led to mortality rates of 40%

.

Exposure to the individual compounds and to 0.33 mg/mL DSS with 39.4 µg/L DSP did not seem to affect dark-light transition response (Fig. 2.63, Table 2.19). However, larvae co-exposed with 0.33 mg/mL DSS and 132 µg/L DSP displayed a sharp increase in overall movement from 10 minutes from the start of observations. When comparing these larvae on a plate-by-plate basis, it is obvious that there was a large variability in response for individual larvae, with some being a lot more active than others (Appendix D).

This large increase was also seen in the distance travelled and speed of larvae co-exposed to DSS and 132 µg/L DSP compared to controls and larvae exposed to 0.33 mg/mL DSS alone. Larvae co-exposed with 0.33 mg/mL DSS and 132 µg/L DSP showed a decrease in drifting movements when compared with larvae exposed to 0.33 mg/mL DSS alone, back to levels comparable with controls.

As seen in previous experiments, zebrafish larvae exposed to 0.33 mg/mL DSS alone significantly prefer to spend time in the outer area of the well versus the central area, swimming significantly less in the centre of the well (Fig. 2.65a). The opposite was seen in larvae exposed to DSP alone, which show a clear preference for the central area of the well (Fig. 2.65). Larvae co-exposed to DSS and DSP display an increased preference for the outer area compared to controls. However, this preference was at levels comparable to those seen in larvae exposed to DSS, which indicates that DSP did not rescue the behaviour alteration caused by DSS.

As shown previously, neutrophil infiltration in the gut was significantly higher in larvae exposed to 0.33 mg/mL DSS. Co-exposure with DSS and 39.4 or 132 µg/L DSP fully recovers this effect, with neutrophil infiltration decreasing to levels comparable with controls.

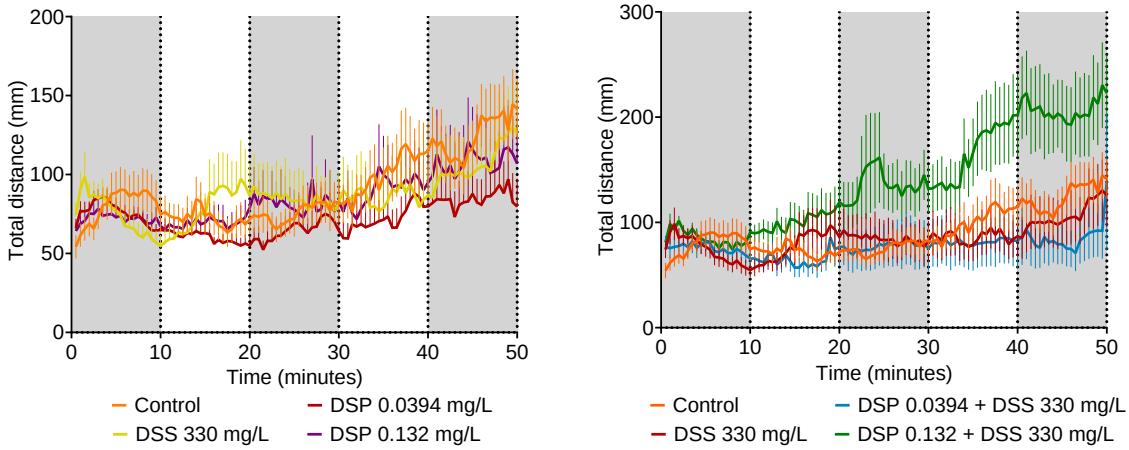
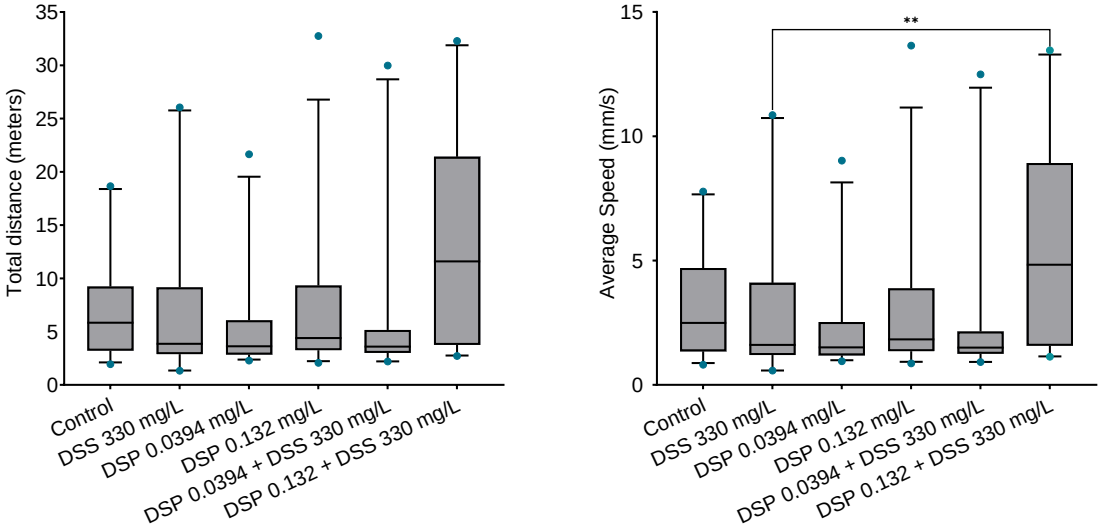


Figure 2.63 – Effect of alternating light–dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 10-day single exposure to 0.33 mg/mL DSS, 39.4 µg/L DSP or 132 µg/L DSP, and two co-exposures with 0.33 mg/mL DSS and 39.4 and 132 µg/L DSP. Free swimming hatched larvae were introduced to the dilutions from 3dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. The statistical methods applied are outlined in the methods section (2.3.8). Data are presented as the mean and standard error of distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=27 (Control), 26 (DSS 0.33 mg/mL), 29 (DSP 3.94 µg/mL), 28 (DSP 13.2 µg/mL) 21 (DSP 3.94 µg/mL + DSS 0.33 mg/mL; DSP 13.2 µg/mL + DSS 0.33 mg/mL) larvae per group. While in most exposure groups dark-light transition response was not affected, larvae co-exposed with 0.33 mg/mL DSS and 132 µg/L DSP displayed a sharp increase in overall movement from 10 minutes from the start of observations.

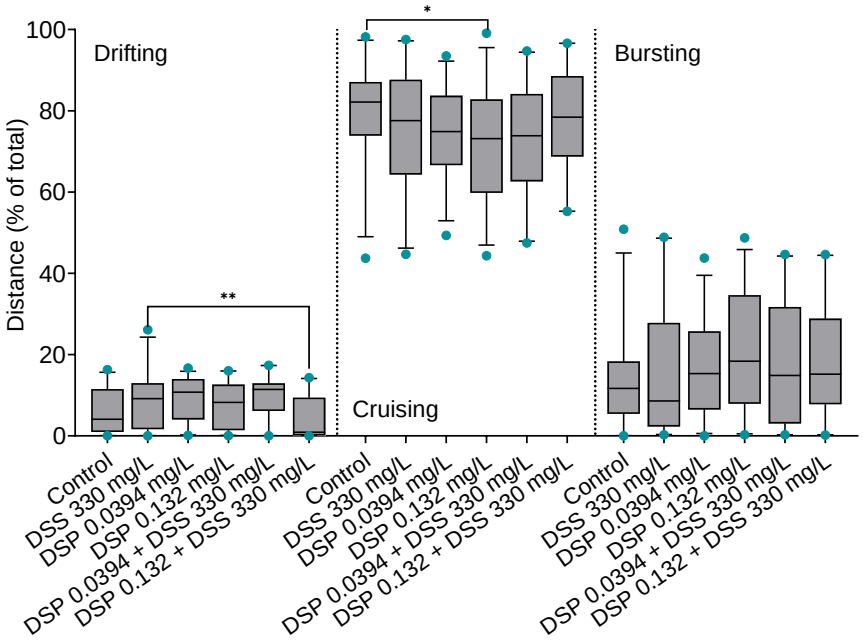
Table 2.19 – **Number of time points with statistically significant difference compared to control for data in Fig. 2.63.** Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). N=27 (Control), 26 (DSS 0.33 mg/mL), 29 (DSP 3.94 µg/mL), 28 (DSP 13.2 µg/mL) 21 (DSP 3.94 µg/mL + DSS 0.33 mg/mL; DSP 13.2 µg/mL + DSS 0.33 mg/mL) larvae per group.

	Control vs. DSS 330 mg/L	Control vs. DSP 0.0394 mg/L	Control vs. DSP 0.132 mg/L	Control vs. DSP 0.0394 + DSS 330 mg/L	Control vs. DSP 0.132 + DSS 330 mg/L	DSS 330 mg/L vs. DSP 0.0394 + DSS 330 mg/L	DSS 330 mg/L vs. DSP 0.132 + DSS 330 mg/L
Time points with statistically significant difference *vs.* control	0	0	0	0	27	0	33



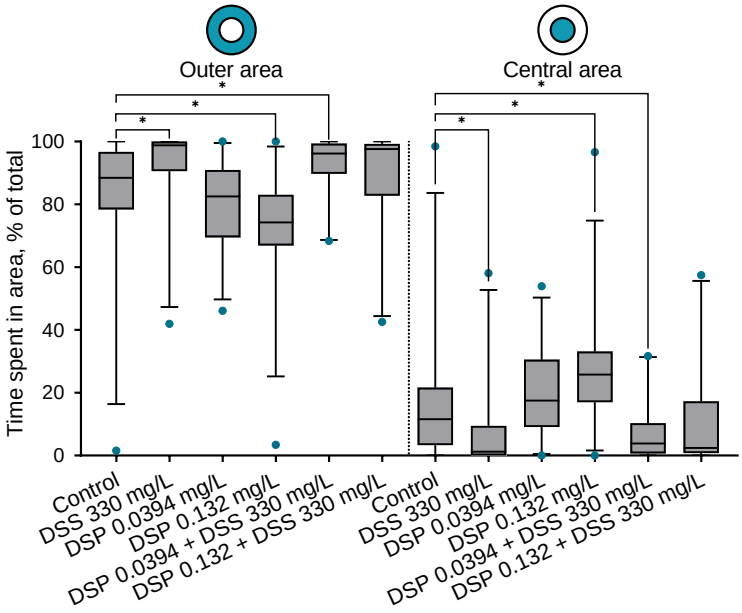
(a) Total distance travelled by larvae (meters)

(b) Average speed of larvae (mm/s)

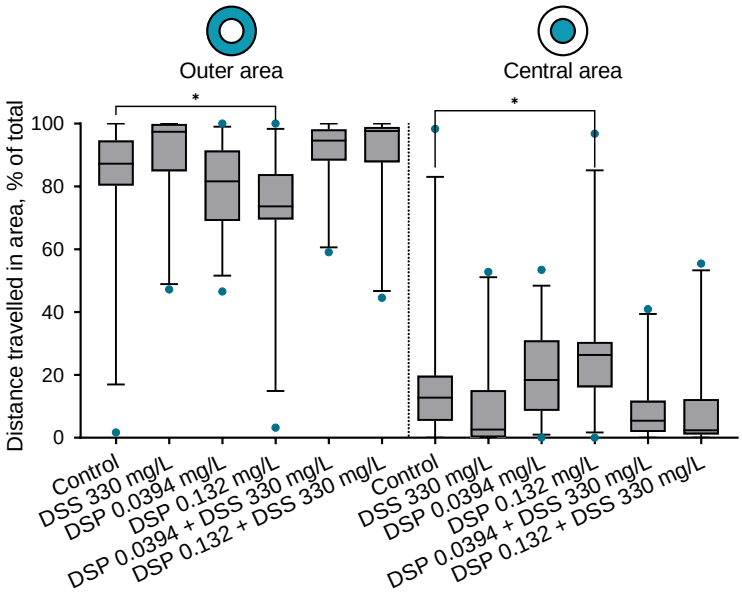


(c) Distance travelled at different speed thresholds

Figure 2.64 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 10-day single exposure to 0.33 mg/mL DSS, 39.4 µg/L DSP or 132 µg/L DSP, and two co-exposures with 0.33 mg/mL DSS and 39.4 and 132 µg/L DSP. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements $>$5mm/s are considered bursting, and $<$1 mm/s are considered drifting. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. N=27 (Control), 26 (DSS 0.33 mg/mL), 29 (DSP 3.94 µg/mL), 28 (DSP 13.2 µg/mL) 21 (DSP 3.94 µg/mL + DSS 0.33 mg/mL; DSP 13.2 µg/mL + DSS 0.33 mg/mL) larvae per group. Larvae co-exposed to 0.33 mg/mL DSS and 132 µg/L DSP showed a increase in the distance travelled and speed of when compared to controls and larvae exposed to 0.33 mg/mL DSS alone. Larvae co-exposed with 0.33 mg/mL DSS and 132 µg/L DSP showed a decrease in drifting movements when compared with larvae exposed to 0.33 mg/mL DSS alone, back to levels comparable with controls.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time



(b) Distance travelled by larvae in the central and outer area of the well

Figure 2.65 – Assessment of behavioural differences in Tg(mpx:GFP)i114 larval zebrafish after 10-day exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period was excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=15 (Control), 23 (DSS 0.33 mg/mL), 15 (DSP 3.94 µg/mL), 16 (DSP 13.2 µg/mL) 14 (DSP 3.94 µg/mL + DSS 0.33 mg/mL; DSP 13.2 µg/mL + DSS 0.33 mg/mL) larvae per group. Larvae exposed to 0.33 mg/mL DSS spent significantly more time in the outer area of the well versus the central area (a), and swim significantly less in the centre of the well (b). Larvae exposed to DSP alone, show preference for the central area of the well. Larvae co-exposed to DSS and DSP, display increased preference for the outer when compared to controls.

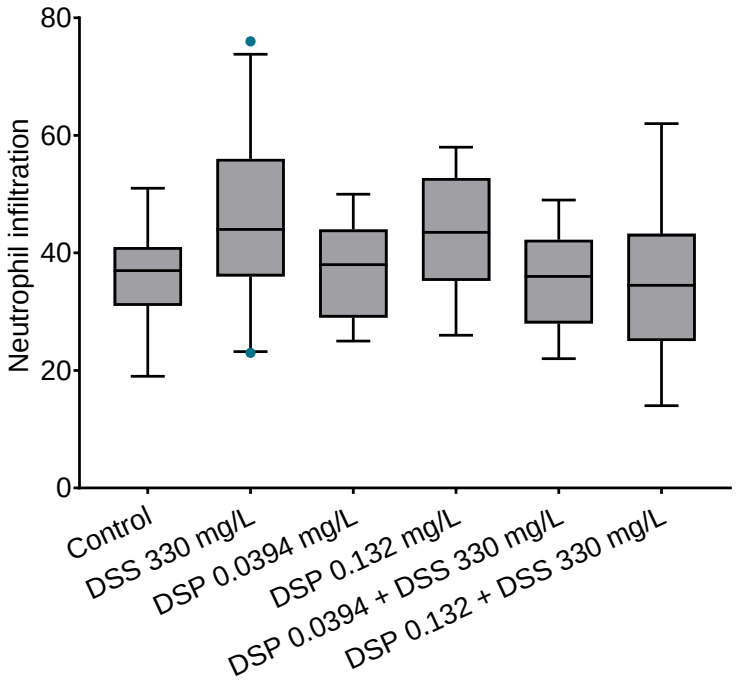


Figure 2.66 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (area as seen demarcated in Fig. 2.4) in Tg(mpx:GFP)i114 larval zebrafish after 10-day single exposure to 0.33 mg/mL DSS, 39.4 µg/L DSP or 132 µg/L DSP, and two co-exposures with 0.33 mg/mL DSS and 39.4 and 132 µg/L DSP. Feeding started at 5dpf. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. N=27 (Control), 26 (DSS 0.33 mg/mL), 29 (DSP 3.94 µg/mL), 28 (DSP 13.2 µg/mL) 21 (DSP 3.94 µg/mL + DSS 0.33 mg/mL; DSP 13.2 µg/mL + DSS 0.33 mg/mL) larvae per group. Exposure to 0.33 mg/mL DSS significantly increased neutrophil infiltration in the gut *vs.* control larvae. Co-exposure with 0.33 mg/mL DSS and 39.4 or 132 µg/L DSP resulted in neutrophil infiltration levels comparable with controls.

2.5 Discussion

The experimental work described in this chapter was aimed at characterising the behavioural effects of pro-inflammatory stimuli in zebrafish larvae using a combination of behavioural tests and immune *in vivo* imaging techniques. The results showed that, of the four different inflammatory treatments, DSS was able to induce both intestinal inflammation and behavioural disruption in zebrafish larvae similar to rodents (Nyuyki et al., 2018; Ko et al., 2020). This suggests that DSS could be a valuable tool for the controlled stimulation of chemical-induced inflammation across species. Nonetheless, the experiments described in this section also present several important challenges that must be taken into account in this field of research.

Table 2.20 contains a summary of behaviour perturbation results in zebrafish larvae exposed to DSS at different concentrations for 2 or 10 days, across 6 experiments.

Table 2.20 – Summary of behaviour perturbation results in zebrafish larvae exposed to DSS at different concentrations for 2 or 10 days. Green indicates a significant change versus controls.

Exposure duration (days)	Experiment ID	Zebrafish line	Tested DSS concentration (mg/L)	Light-Dark response	Distance	Speed	Thigmotaxis
2	DSS pilot	Wild-type	4.5				
2	DSS 1	Tg(mpx:GFP)	2.5				
2	DSS pilot	Wild-type	2.25				
2	DSS 1	Tg(mpx:GFP)	1.25				
2	DSS 1	Tg(mpx:GFP)	0.65				
10	DSS 3	Tg(mpx:GFP)	0.3				
10	DSS 5	Wild-type	0.3				
10	DSS 3	Tg(mpx:GFP)	0.1				
10	DSS 3	Tg(mpeg1:mCherry)	0.1				
10	DSS 4	Wild-type	0.1				
10	DSS 3	Tg(mpx:GFP)	0.03				
10	DSS 3	Tg(mpeg1:mCherry)	0.03				
10	DSS 3	Tg(mpx:GFP)	0.01				
10	DSS 3	Tg(mpeg1:mCherry)	0.01				
10	DSS 3	Tg(mpeg1:mCherry)	0.003				
10	DSS 3	Tg(mpx:GFP)	0.001				
10	DSS 3	Tg(mpeg1:mCherry)	0.001				

Zebrafish larvae at 5 dpf exhibit a specific movement pattern in response to alternating light and dark conditions, characterized by low activity in the light phase and high activity in the dark. This behaviour is remarkably consistent, and it has been investigated in many previous studies that tested the effects of both chemical and non-chemical stressors on zebrafish larvae (Li et al., 2014; Legradi et al., 2015; Basnet

et al., 2019). These locomotor activities are contingent upon the proper functioning and development of the brain, nervous system, and visual pathways (Ali et al., 2012; Bilotta, 2000). The increased locomotor activity observed during the light-dark transition is attributed to increased stress/anxiety levels in the larvae (Irons et al., 2010; MacPhail et al., 2009; Vignet et al., 2013). This change in behaviour was observed in our 5 dpf experiments in control larvae, with DSS consistently disrupting this response. However, the same response was not observed in 13 dpf larvae, in controls and after 10 days exposure to DSS. At 13 dpf the response to dark/light stimuli differs from the response at 5 dpf, lacking the obvious decrease in movement when larvae are exposed to light. Gould (2022) has reported the same shift in repsonse in 28-day and 42-day-old zebrafish. There are other reports that the differences in activity levels between the light and dark phases are gradually reduced in zebrafish from 4 to 10dpf (Kristofco et al., 2016). Existing literature has also established that while zebrafish larvae exhibit a preference for light zones, juveniles and adult animals prefer dark zones (Kalueff et al., 2014; Kysil et al., 2017; Bai et al., 2016), although the precise timing of this transition is still uncertain (Petersen et al., 2022). However, the exact reasons for the variation in fish responses to changes in lighting conditions at different life stages, and the differences in these responses across species, remain unknown.

In addition to assessing locomotory activity under light and dark conditions, another important behaviour considered in this project was thigmotaxis, which refers to an animal's tendency to move in close proximity with a vertical surface (Basnet et al., 2019). In this experiment, thigmotaxis was observed in the form of zebrafish larvae moving along the walls of the individual well of the plate where they were housed. Important features of thigmotaxis are the avoidance of the central part of the area housing the animal, and preference towards the edge or periphery. Thigmotaxis is a well-conserved behaviour across species, including mice and humans (Gromer et al., 2021), and is generally considered to be anxiety-driven. Previous studies have demonstrated that thigmotaxis in zebrafish larvae can be modulated by anxiolytic drugs, such as diazepam, and anxiogenic drugs, such as caffeine and pentylenetetrazole, confirming the biological and translational value of this behavioural response (Schnörr et al.,

2012a). Overall, both the response to light-dark challenges and thigmotaxis provided a useful range of biologically meaningful parameters for the detection of inflammation-induced perturbation of zebrafish behaviour.

TNBS was the first pro-inflammatory treatment tested in this study. Treatment with this compound induced the expected increase in neutrophil infiltration in the intestinal area in addition to an overall decrease in locomotory activity. TNBS is widely used to induce colitis in rodent models, and previous studies have reported that TNBS-induced colitis in mice is associated with the perturbation of behaviour, quantified using anxiety-like and depressive-like behavioural tests (Haj-Mirzaian et al., 2017; Emge et al., 2016; Chen et al., 2015; Vecchiarelli et al., 2021). These behavioural alterations have also been associated with altered hippocampal glutamatergic transmission (Riazi et al., 2015). The data generated in our studies showed a possible concordance with the rodent data discussed above. However, TNBS was not considered as an appropriate candidate for this project as its use appears to be incompatible with co-exposure with other pharmacological agents, which are oxidized by TNBS, and the treatment was excluded from further studies. Nonetheless, TNBS may remain a viable option for future studies that do not require co-exposure with other compounds.

This study examined two non-chemical treatments known to induce behavioural changes in mammals. LPS is commonly used to induce sickness behaviour in rodent models, characterized by anhedonia, lethargy, loss of appetite, decreased locomotory activity, increased anxiety, and sleepiness (Bassi et al., 2012; Shishkina et al., 2020; Lasselin et al., 2020). Notably, the doses of LPS used to induce a sickness response in mice or rats are about 10,000–100,000 times higher than those administered to humans (Lasselin et al., 2020). This considerable difference in LPS effective dose across species may suggest that the waterborne exposure to LPS used in the present study may have not produced a concentration high enough plasma LPS concentration to elicit significant immune effects, thus explaining the absence of behavioural effects.

The second non-chemical pro-inflammatory treatment tested in our project was high-fat/high-cholesterol diet. In rodents, this treatment has been linked with a diverse set of behavioural alterations, including increased anxiety and decreased locomotory

activity (Keleher et al., 2018; Han et al., 2021; Noronha et al., 2019), associated with the dysregulation of dopamine pathways (Han et al., 2021). However, our zebrafish experiments did not highlight any significant change in behaviour. It is important to consider that rodent studies typically involve the use of a high-fat diet for periods ranging from 3 to 10 weeks, whereas the zebrafish larvae in our study were subjected to a modified diet for just 10 days, of which part was characterized by a mix of endogenous and exogenous feeding. Although a high-cholesterol diet has been demonstrated to induce intestinal inflammation in zebrafish after just 6 hours (Progatzky et al., 2014), the short duration of the treatment was likely the major cause underlying the lack of significant effects. Given the translational significance of high-fat diets, extending the duration of diet administration in future studies would hold considerable importance. It is plausible to hypothesise that extended treatment durations may elicit robust biological responses capable of influencing behavioural phenotypes. This hypothesis finds support in a recent study by Picolo et al. (2021), which revealed that adult zebrafish administered a high-fat diet for 16 days exhibited heightened aggression, anxiety-like behaviours, and impaired memory formation, while their locomotory and exploratory activity remained unaffected.

Copper sulphate is known to induce behavioural effects in rodents but this is believed to be due to its toxic impact on the brain and other organs rather than any subtle inflammatory responses, as reported by Lamtai et al. (2020). In the present project, copper sulphate was selected for the demonstrated ability to induce inflammation in the zebrafish neuromasts after waterborne exposure, and the consequent disruption of the lateral line functionality. However, the imaging facilities available during the project prevented the optimization of the analytical process, leading to the exclusion of this compound from the experimental pipeline.

DSS was the only pro-inflammatory treatment considered suitable for full characterization. This compound is a well-characterized pharmacological agent used to induce colitis in rodent models, an effect that has been associated with reproducible alterations of anxiety- and depressive-like behaviours, such as decreased locomotory activity and increased thighmotaxis (Dempsey et al., 2019). In the present study, a

comparable change in thigmotaxis was observed only occasionally in the three different experiments performed, involving 23 different treatment groups, excluding the controls.

DSS affected larvae's preference for the central or outer area of the well in distinct ways. While 13 dpf larvae displayed classic thigmotactic behaviour, preferring the outer area of the well, 5 dpf larvae showed a slight increase in preference for the central area. However, this effect was only observed in one concentration and did not follow a dose-response. The biological relevance of this observation remains unclear. While experiments were not conducted to replicate this specific result after 48h exposure, this effect was observed consistently in larvae exposed to a 0.33mg/mL DSS concentration for 10 days.

Conversely, the perturbation of locomotor activity during the light-dark challenge was more frequently observed across different zebrafish lines (as documented in Table 2.20). However, the reproducibility of these effects was only in the range of 55-60%. These effects included both an increase and a decrease in locomotor activity in different cases, which complicated the interpretation of the data. In rodents, the effects of DSS generally lead to a decrease in locomotion and an increase in anxiety-related behaviours (Dempsey et al., 2019; Gadotti et al., 2019). However, a quantitative assessment of the degree of DSS-induced effects in rodents is not available. Some studies performed dose-response experiments, indicating that after 6 weeks of treatments, doses of DSS between 0.25–1% (in drinking water) caused diarrhea and blood in stools whereas, after 12 weeks, 0.5% DSS induced chronic and sustained infiltration of inflammatory cells in the gastrointestinal tract (Ghattamaneni et al., 2019), in addition to increased production of pro-inflammatory cytokines, hyperemia, ulcerations, moderate to severe submucosal oedema, and lesions (Randhawa et al., 2014). Typically, shorter-term experiments involve exposure to 2 to 5% DSS in drinking water for 5 to 9 days (Randhawa et al., 2014). To enable a proper comparison between rodent studies and our zebrafish studies, two crucial factors that must be taken into account are the dose and exposure duration. The maximum concentration of DSS used in our longer-term studies with zebrafish was 0.33 mg/mL. This concentration is 60 times

lower than the lowest concentration typically used in drinking water to induce colitis in rodents in the short term (2%, 20 mg/mL) and measure related behavioural effects.

Assuming that DSS has no effect on water intake, in a typical experiment performed using 2% DSS in drinking water, a rat may be expected to ingest about 30 mL of water per day (9-12 mL per 100 g of body weight) (Vasilev et al., 2021). This would translate into 60 mg of DSS ingested per day, which would directly reach the site of interest, *i.e.*, the intestine. In contrast to rodents, zebrafish waterborne exposure is continuous and not limited to a single drinking event. Furthermore, the rate of DSS uptake from water into the zebrafish through their skin and gills remains unknown. Unlike rodents, the route of waterborne exposure for zebrafish means that the intestine is not the sole site of action for DSS. This compound is likely to induce pro-inflammatory effects in other parts of the organism as well, which could explain the narrow safety window observed for DSS during the project and the instances of unexpected mortality. Due to the need for a non-lethal concentration of DSS, we established a maximum concentration of 0.33 mg/mL to be utilized in 10-day DSS exposure experiments with zebrafish. However, it is plausible to hypothesize that this concentration may induce biological effects that are less intense than those observed with 2-5% DSS in rodents. It is worth noting that treatment with DSS can also result in mortality in rodents, although this parameter is only reported in 28% of DSS-induced colitis papers (Bramhall et al., 2015). This biased reporting has contributed to the challenges encountered in optimizing the zebrafish exposure protocol, as it has given the false impression that very high doses of DSS in mice are highly safe and not associated with increased mortality. In reality, the limited available data in the literature indicate that the mortality rate may be considerable. For instance, Zaki et al. (2010) reported a 20% mortality after treating male C57BL/6 mice with 2.5% DSS in drinking water. Further research aimed at characterizing uptake and internal concentrations of DSS in zebrafish larvae and addressing the mortality reporting bias in rodent studies could help improve the overall study design and facilitate data interpretation.

From a histopathological perspective, DSS induced highly reproducible effects characterized by a consistent increase in the infiltration of neutrophils in the mid and

posterior intestine of zebrafish larvae exposed for both 2 days and 10 days. While previous studies have not reported on the behaviour of zebrafish exposed to DSS, the literature indicates that DSS immersion consistently induces an inflammatory response, specifically increased neutrophil infiltration in the intestine (Oehlers et al., 2013, 2012; Chuang et al., 2019). The most accepted mechanisms of DSS-induced intestinal inflammation involve the disruption of the intestinal barrier, which increases its permeability, leading to the entry of luminal bacteria and associated antigens into the mucosa, and allowing the dissemination of pro-inflammatory intestinal contents into underlying tissue (Kiesler et al., 2015; Perše and Cerar, 2012; Wirtz et al., 2007; Okayasu et al., 1990; Poritz et al., 2007; Kim et al., 2012; Samak et al., 2015; Eichele and Kharbanda, 2017). This process is coupled with increased production of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, and increased toll-like receptor 4 (TLR4) gene expression (Randhawa et al., 2014; Perše and Cerar, 2012), which are main components of the inflammatory pathways linked to depression (Fig. 1.3). These findings suggest that this model may provide a useful tool for studying the relationship between inflammation and behaviour.

To validate the inflammatory nature of the behavioural alterations observed in zebrafish larvae exposed to DSS, we co-exposed larvae to both DSS and dexamethasone, a potent steroidal anti-inflammatory drug that acts as glucocorticoid receptor agonist. However, in our behavioural tests, the overall locomotory activity displayed by larvae co-exposed to DSS and dexamethasone was highly variable. As previously seen with DSS alone, larvae co-exposed to DSS and dexamethasone displayed an increased preference for the outer area compared to controls, indicating that dexamethasone did not recover the behavioural alteration caused by DSS. Larvae exposed to dexamethasone alone showed a clear preference for the central area of the well, which could be interpreted as an anti-anxiolytic effect. However, further evidence is needed to confirm this interpretation. The co-exposure of larvae to both DSS and dexamethasone led to a recovery of the effect of DSS on inflammation, with neutrophil infiltration decreasing to levels comparable to the controls. This recovery is consistent with dexamethasone's mechanism of action of inhibiting macrophage and neutrophil

recruitment to the injury site. However, the results obtained in behavioural tests do not suggest a direct effect of dexamethasone on behaviour.

Although selective cyclooxygenase (COX)-2 and non-selective COX inhibitor NSAIDs have previously been studied as co-treatments for major depression 1.11), evidence on the effect of glucocorticoids on behaviour is limited to the previously described use of dexamethasone as a diagnostic tool for depression (see section 2.2).

Despite the fact that our study demonstrated an association between DSS- induced inflammation in zebrafish and disruption of behavioural phenotypes, it is important to note that the observed neutrophil infiltration represents only a small part of a much wider inflammatory response and questions still remain regarding the causal mechanisms behind the behavioural alterations observed. The involvement of the hypothalamic-pituitary-adrenal (HPA) axis, glucocorticoid and cortisol levels, specific inflammatory markers, and neurotransmitters, among other factors, may be key to better understanding the link between inflammation and behavioural changes in the zebrafish model.

Due to technical and resource constraints, RNAseq analysis of exposed larvae was planned but not performed. Investigating the differential expression patterns in zebrafish larvae exposed to these drugs through transcriptomics may offer additional insights into the *in vivo* effects and elucidate relevant pathways that may be involved in a chemical-induced inflammation of behavioural effects.

The high levels of variability observed not only between our own experiments and between individual larvae but also between our results and the results reported in the literature are not unexpected. It has been previously reported that fish behaviour exhibits a high degree of individual variability (Tanoue et al., 2019; Margiotta-Casaluci et al., 2014; Huerta et al., 2016). While increasing the number of fish used in an experiment has been suggested as a way to deal with the high degree of variability and increase statistical power, this approach raises ethical concerns. Moreover, the observed variability in the effects of psychoactive drugs on fish behaviour has been noted in experiments that involved the use of 5 to 16 fish per treatment group (Tanoue et al., 2019). The question remains as to how many fish per treatment group are needed to

account for this variability. To address the observed variability in the effects of psychoactive drugs on fish behaviour, we conducted exposures with zebrafish larvae up to 5 dpf as they do not carry the ethical and legal considerations of adult fish. Additionally, we pooled data from several experiments to increase the sample size per treatment group. However, even with these measures, variability in behaviour was still present. Our sample sizes in behavioural experiments varied in some cases due to mortality, and even in the control group with 72 pooled larvae from three separate experiments, we observed a considerable amount of variability in behaviour.

A more effective approach would involve improving our understanding of fish behaviour. It is important to investigate why certain fish display high levels of activity while others do not, why some fish exhibit a greater response to psychoactive drugs than others, and the degree of repeatability of these behaviours. Investigating the behaviour of control fish and using cluster analysis to group fish into categories could provide a more robust statistical analysis that accounts for inter-individual variability.

However, the contradictory results in the literature and difficulty in interpretation persist. In some cases, different papers report contradictory results, making it difficult to determine which results are reproducible. For instance, while one study reported pronounced adverse effects of the antidepressant venlafaxine on young fish (Thompson et al., 2017), another study observed no effects of the same drug over a full fish life cycle (Parrott and Metcalfe, 2017). Despite the use of robust experimental designs, it is possible to obtain an equivocal set of behavioural data, making interpretation difficult even for a panel of experts, as reported by Tanoue et al. (2019). In some cases, behavioural tests for certain drugs, such as fluoxetine produce clear and reproducible results across multiple experiments (Tanoue et al., 2019; Margiotta-Casaluci et al., 2014; Cachat et al., 2010; Ansai et al., 2016). However, in other cases, results can be challenging to interpret, as with tramadol, where experts have reached different conclusions based on the same data, suggesting that different statistical approaches might lead to varying outcomes Tanoue et al. (2019). Furthermore, it has been noted that different authors report varying concentrations of psychoactive drugs that are required to affect fish behaviour, ranging from low ng/L to high µg/L Sumpter et al. (2014).

Furthermore, it has been suggested that the interpretation of behavioural data can be influenced by confirmation bias, which is the tendency to interpret or recall information in a way that confirms pre-existing beliefs Tanoue et al. (2019). For example, when examining an apparent effect of the lowest concentration of a chemical that is not seen at higher concentrations, confirmation bias may lead to the conclusion that the results follow a non-monotonic dose-response relationship if one believes that low concentrations of the chemical can cause effects not produced by higher concentrations. However, if one believes that low doses cannot cause an effect, the conclusion from the same data could be that the apparent effect of the lowest concentration tested is likely an artefact due to chance and may not be reproducible Tanoue et al. (2019).

Variability in behaviour is not exclusive to fish, with results of rodent behavioural tests varying depending on the person conducting the experiment (Chesler et al., 2002), the laboratory in which the experiments are performed (Crabbe et al., 1999), and environmental factors such as animal housing (Richter et al., 2010; Hånell and Marklund, 2014).

Sharp differences in fish behaviour as they age can also pose challenges to data interpretation. For instance, changes in the preference from dark to light and alterations in the degree of thigmotaxis observed in zebrafish larvae from 5 to 13 pdf raises questions about the timing of these changes, their reproducibility, and any other changes that may occur in zebrafish behaviour during later life stages.

Additionally, the observed mortality rates in our experiments occasionally differed not only from what is reported in the literature but also between our own experiments, including in the control groups. The lack of published data on not only baseline zebrafish behaviour but also the natural mortality rates at different life stages, especially during the critical stage of feeding initiation, is a significant limitation in better understanding the variations observed between larvae and experiments.

Consideration should also be given to potential issues arising from the isolation of zebrafish in individual wells of 24-well plates during the exposure and behavior-tracking period. while transparent well plates allow the visual perception of other larvae, zebrafish are inherently social animals, and their behaviour in isolation may differ from

their natural social interactions. Studies have shown that extended periods of isolation, such as from 6 dpf to 6 months, can lead to profound alterations in brain structure and function (Eachus et al., 2021), the implications of short-term isolation on larval behaviour and physiology remain uncertain. Nonetheless, this approach is standard in behavioural, toxicological, and drug discovery investigations (OECD, 2013; Batista-Filho et al., 2021; Legradi et al., 2015; Ellis and Soanes, 2012), suggesting an absence of deleterious effects resulting from this isolation practice. Overall, the numerous benefits for precise data collection and analysis derived from isolating zebrafish, outweigh any potential concerns. Future advancements in technology for behaviour tracking may offer opportunities to study zebrafish behaviour in pairs or groups, mitigating any concerns regarding the impact of isolation on behaviour and brain development.

It's worth noting that a major limitation of this study is the lack of analysis of drug concentrations in water, fish plasma, or tissue. It has been reported that there is a degree of inter-individual variability in plasma concentrations for fish from the same tank exposed to psychoactive drugs (Huerta et al., 2016; Margiotta-Casaluci et al., 2014), which could explain, in part, the observed variability in zebrafish behaviour and mortality rates.

Furthermore, the identification of the exposure groups in the experiments had the potential to introduce unintentional bias and influence the interpretation of results. However, despite the absence of blinding during the behaviour component of the experiments, the analysis was conducted using Zebralab software, an automated tool designed to objectively quantify and assess various behavioural parameters. Since the software analyzes the data consistently, the risk of bias during this stage is minimized. Furthermore, during the acquisition of microscopy images, files were deliberately labelled without exposure group information. This blinding step ensured that, during manual analysis, knowledge of the specific exposure groups did not inadvertently introduce bias, thereby maintaining objectivity in the interpretation of the data.

An equally noteworthy limitation is that the quantification of neutrophil infiltration was conducted by the same individual. To mitigate potential bias and error that this can introduce, replicates were blinded, a control was established, and predefined analysis

criteria were consistently followed. While these measures can mitigate some issues, they do not eliminate the risk entirely. Inter-rater reliability, which involves having multiple analysts independently assess the same set of images, could provide a more robust assessment of the analysis's consistency, as well as identify and address any discrepancies or potential bias introduced by a single analyst. Having a second person confirm the results could enhance the reliability and validity of the findings, offering a more objective and rigorous evaluation of the data.

2.6 Conclusion

By employing a combination of behavioural phenotyping and immune *in vivo* imaging techniques, our study demonstrates that DSS-induced inflammation in zebrafish consistently disrupts behavioural phenotypes. However, it is essential to note that neutrophil infiltration represents only a small fraction of a broader inflammatory response. The underlying mechanisms behind the behavioural alterations in response to inflammation remain unclear, and questions regarding the involvement of the HPA axis, glucocorticoid and cortisol levels, specific inflammatory markers, and neurotransmitters require further investigation.

Investigating the differential expression patterns in zebrafish larvae exposed to pro-inflammatory stimuli may provide additional insights into specific inflammatory markers and aid in exploring the inflammatory theory of behavioural alteration in mental health disorders like depression. Our preserved larvae samples can facilitate future studies of expression data, which could help validate the inflammatory results observed in the imaging experiments.

Further improving the understanding of variability in fish behaviour, activity levels, and response to compounds is crucial to establish the reproducibility of these experiments. Additionally, developmental shifts in anxiety-related behaviour must be further studied and considered when interpreting behavioural responses to experimental treatments.

Overall, our study provides novel insights into the effects of DSS-induced inflammation on behavioural phenotypes in zebrafish and underscores the importance of

considering variability between individual zebrafish and distinct experiments. Our work suggests that the zebrafish model could serve as a complementary model to investigate the relationship between inflammation and behaviour, although additional research is necessary to validate its use. Our research serves as a stepping stone for future investigations aimed at elucidating the precise mechanisms underlying the relationship between inflammation and behaviour. The novel zebrafish model proposed in this study has the potential to be used in more extensive drug screening activities to identify compounds that can rescue inflammation-induced behaviour before initiating pre-clinical work using higher vertebrate models.

2.7 References

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Chapter 3

Analysis of drug transcriptomic signatures for the comparative assessment of the immunomodulatory potential of antidepressant drugs

Disclaimer *This chapter contains work conducted in collaboration with the Computational Biology Facility at Liverpool University. In Section 3.3.2, the drug selection was performed by me, while the method described section was developed by Arturas Grauslys (Computational Biology Facility at Liverpool University), as were the data extraction from Library of Integrated Network-Based Cellular Signatures (LINCS) L1000 and Reactome and figures, all other tables, analysis, hypothesis, research, discussion and conclusions are my own.*

3.1 Abstract

In recent years, the recognition that inflammation is a fundamental aspect of major depressive disorder (MDD) has prompted extensive research to establish the effectiveness of anti-inflammatory pharmacological interventions for the treatment of depression, alone or in combination with antidepressants. While the causal relationship between immune dysfunction and MDD pathophysiology remains unclear, antidepressants have been shown to affect both immune cells and signalling molecules, leading

to both immunomodulatory and pro/anti-inflammatory effects.

To address the knowledge gap regarding the immunomodulatory potential of antidepressants, the transcriptomic signature data of 21 different antidepressants extracted from the L1000 Connectivity Map database was assessed. Drug-gene interaction data was then used to assess the ability of the compounds of interest to perturb relevant biological pathways hosted in the Reactome Knowledgebase. The results revealed that different classes of antidepressants interact with various pathways, and even within the same class, immunomodulatory action can vary.

Certain markers, such as TNF-alpha and IL-6, have been suggested as predictors of antidepressant effectiveness, while others, like IL-17A, have been suggested as markers of therapy resistance. Our enrichment analysis predicted that these pathways were targets of antidepressant action. It was also found that several antidepressants interact with genes related to the IL-20 signalling pathway, an area of cytokine research that is still relatively unknown. Perturbation databases like the L1000 Connectivity Map offer opportunities for computational drug discovery approaches that extend beyond classical pharmacology. Validating these predicted drug-gene interaction enriched pathways experimentally is crucial, and future research looking at differential gene expression signatures for specific drug-gene interactions linked to immune pathways of interest could shed light on the effects of antidepressants on pro- and anti-inflammatory mechanisms. Overall, this work highlights the complex interaction between antidepressants and the immune system and emphasises the need for personalised medicine considerations in developing more effective treatment strategies for depression.

3.2 Introduction

The clinically proven observation that inflammation is a core feature of major depressive disorder has prompted an intense programme of research aimed at establishing the effectiveness of anti-inflammatory pharmacological strategies for the treatment of depression. Many clinical trials have been carried out to test the efficacy of pharmacological combinations, including antidepressants and nonsteroidal anti-inflammatory

drugs (NSAIDs), with either positive (Pasco et al., 2010; Nery et al., 2008; Muller et al., 2006; Akhondzadeh et al., 2009; Tyring et al., 2006; Mendlewicz et al., 2006), negative (Uher et al., 2012; Fields et al., 2012; Almeida et al., 2012), or even detrimental results (Warner-Schmidt et al., 2011; Gallagher et al., 2012).

In parallel with the pharmacological research, a growing volume of clinical investigations has demonstrated that patients affected by major depressive disorder also display dysregulation of specific immune cells, in addition to altered levels of immune signalling molecules (*e.g.* tumor necrosis factor (TNF)-α, interleukin (IL)6, C-reactive protein (CRP)) (Strawbridge et al., 2015). However, it is not known whether such dysfunction is the cause or consequence of the disease, and to what extent immune dynamics contribute to the disease pathophysiology. For example, early studies suggested that the impaired functioning of the T cell system (such as T cell proliferation capacity, reductions of CD3+ T cells and cluster of differentiation (CD)4+ T helper cells), and natural killer cell (NK) cells (including reduced NK cell numbers and cytotoxicity) may be involved in the aetiology of major depressive disorder (Miller, 2010; Zorrilla et al., 2001). Grosse et al. (2016) confirmed these observations by observing reduced levels of circulating natural regulatory T cells (Treg) in untreated patients, followed by a pronounced increase in Treg cells after antidepressant treatment. In addition, the authors observed that specific subsets of immune cells, such as cytotoxic T cells, NK cells, were also predictive of non-responsiveness to antidepressants.

In addition to direct immunomodulation, antidepressants can also show intrinsic anti-inflammatory properties that may contribute to their efficacy. While the full clinical relevance of the anti-inflammatory effect is not fully clear, cytokine production by immunocompetent cells has been shown to be affected by antidepressants. Clomipramine, imipramine, fluoxetine and citalopram exert varying degrees of inhibition of IL1β, IL-6 and TNF-α, IL-2 and interferon (IFN)-γ (Xia et al., 1996; Sacre et al., 2010). Furthermore, fluoxetine and citalopram treatment selectively inhibit the signalling of endosomal toll-like receptors (TLRs) 3, 7, 8, and 9 (Sacre et al., 2010). The selective serotonin reuptake inhibitors (SSRIs) fluoxetine, sertraline, paroxetine, fluvoxamine and citalopram at concentrations known to occur in the brain with conventional

pharmacological treatment substantially attenuated LPS-induced TNF-α production on microglia (Tynan et al., 2012). Inhibition of protein kinase A (PKA) with ribose-modified cyclic adenosine monophosphates (Rp-cAMPs) attenuated the anti-inflammatory response of the majority of these antidepressants, which could indicate that PKA plays a partial role in the anti-inflammatory effects of SSRIs.

The tricyclic antidepressants (TCAs) imipramine, amitriptyline, maprotiline, and clomipramine displayed anti-inflammatory activity *in vivo* in the carrageenan model of paw inflammation in rats (Sadeghi et al., 2013; Abdel-Salam et al., 2003). In the same rat model, trazodone (Abdel-Salam et al., 2003) and bupropion (Hajhashemi and Khanjani, 2014) significantly inhibited paw oedema. Carrageenan-induced paw oedema in rats is a valid and routine model used to assay anti-inflammatory activity. The probable mechanism of action of this model is three-phasic, with the first phase attributed to the release of histamine and serotonin, the second phase mediated by kinins, and the third phase attributed to the release of prostaglandins and lysosome enzymes (Di Rosa et al., 1971; Hajhashemi and Khanjani, 2014). The effect of bupropion was investigated at the later phases of the inflammatory response, which indicates that the anti-inflammatory effect of the drug is via inhibition of prostaglandin synthesis.

In contrast, bupropion failed to show any anti-inflammatory effect in croton oil-induced ear swelling in mice, a model used to detect the anti-inflammatory potential effects of histamine ligands. These results confirm that the anti-inflammatory effect of bupropion is not mediated by histamine (Hajhashemi and Khanjani, 2014). It has also been reported that amitriptyline, imipramine, and clomipramine appear to be more effective at decreasing inflammation during the early phase of carrageenan oedema, when histamine, 5-hydroxytryptamine (serotonin) (5-HT) and bradykinin are the mediators involved (Abdel-Salam et al., 2003). Meanwhile, trazodone, like bupropion, appeared to be effective at later stages of carrageenan oedema (Abdel-Salam et al., 2003), strengthening the hypothesis that different mechanisms are likely to be involved in the anti-inflammatory reaction of different antidepressants.

Other studies support the hypothesis that the anti-inflammatory effect of clomipramine and fluoxetine is linked to changes in local mediator release. Con-

centrations of prostaglandin E2 (PGE2) and substance P - a peptide released from the peripheral endings of small unmyelinated afferent C-fibers that has been demonstrated to mediate neurogenic inflammation in rat skin - in the inflammatory exudate were reduced following the administration of these drugs (Bianchi et al., 1995). Furthermore, fluoxetine significantly and dose-dependently reduced the swelling induced by the injection of 10% brewer's yeast suspension in the hind paw of rats. Both adrenalectomy and hypophysectomy prevented the effect of fluoxetine, while pretreatment with the corticotropin-releasing hormone antagonist a-helical corticotropin-releasing hormone (CRH)-(9-41) did not interfere with the anti-inflammatory action of fluoxetine. These effects suggest an involvement of the hypothalamic-pituitary-adrenal (HPA) axis in the anti-inflammatory action of this drug (Bianchi et al., 1994).

It is worth noting that some of these results indicate that, under certain conditions, SSRIs possess the capacity to exert both pro- and anti-inflammatory properties (Tynan et al., 2012; Abdel-Salam et al., 2003). While, as mentioned above, the SSRIs fluoxetine, sertraline, paroxetine, fluvoxamine, and citalopram can decrease TNF-α production, longer exposures to low concentrations of these antidepressants lead to a state in which these antidepressants were moderately pro-inflammatory (Tynan et al., 2012). Equally, in carrageenan-induced paw oedema in rats, fluoxetine displayed anti-inflammatory activity through dose-related reduction of oedema (Abdel-Salam et al., 2003), while sertraline caused dose-dependent enhancement of oedema. Sertraline is a potent inhibitor of 5-HT reuptake (Sánchez and Hyttel, 1999), making it unlikely for modulation of serotonergic neurotransmission to be operative in the anti-inflammatory properties described for fluoxetine and the other antidepressants. The marked anti-inflammatory effect of amitriptyline, which has similar reuptake inhibitory potencies for 5-HT and noradrenaline (NA) (Sánchez and Hyttel, 1999), supports this theory.

Several studies have reported a dissociation of the anti-inflammatory and anti-nociceptive effects of antidepressants. Amitriptyline, imipramine and clomipramine exerted significant anti-oedema effects at doses which had no or slight effect on nociceptive thresholds to electrical stimuli, while doses of trazodone which exerted notable anti-nociception had no anti-inflammatory effect. On the other hand, sertraline exac-

erbated oedema in carrageenan-induced inflammation but displayed anti-nociceptive effects in a test involving electrical stimulation of the tail (Abdel-Salam et al., 2003; Bianchi et al., 1994).

Historically, the theoretical justification for the use of these drugs to treat depression is provided by the mono-amine theory, which held that depression arises from a deficit in brain mono-amines, such as serotonin and the catecholamines dopamine, adrenaline, and noradrenaline (Heninger et al., 1996; Tynan et al., 2012). However, this theory is insufficient to explain the therapeutic effects exerted by antidepressants (Heninger et al., 1996; Tynan et al., 2012; Lacasse and Leo, 2005) and, as mentioned in 1, there is now abundant evidence to support the neuro-inflammatory theory of depression. Based on this theory and the evidence presented above, it can be hypothesised that drugs currently prescribed on the basis of their ability to alter mono-amine reuptake might owe at least some of their effectiveness as antidepressants to their ability to inhibit inflammatory activity in the brain (Tynan et al., 2012).

Demystifying the mechanisms behind how different antidepressants exert their anti-inflammatory effects and which ones have anti-inflammatory potential could not only help elucidate the inflammatory component of depressive disorders, but also provide a tool to help select the correct drug for use in depression or pain management. Could The response to certain antidepressants but not others indicate the presence of an inflammatory component of depression? Does the treatment of depressive symptoms with co-administration with anti-inflammatory drugs support this hypothesis? Exactly which mechanisms underlay inflammation in depression?

The research discussed above indicates that antidepressants can affect both immune cells and their signalling molecules, leading to both immunomodulatory and pro/anti-inflammatory effects (Szałach et al., 2019; Antonioli et al., 2012). However, the full set of mechanisms underlying these effects is currently unknown. Due to the involvement of immune responses in both the aetiology of depression and the responsiveness to antidepressants, there is an urgent need to generate a comparative evaluation of the intrinsic ability of antidepressants to modulate the immune system. In turn, this knowledge could support the design of more effective treatment strategies,

potentially based on personalised medicine considerations. The immunomodulatory properties of a small number of antidepressants (*e.g.* fluoxetine, paroxetine, venlafaxine, desvenlafaxine, imipramine, amitriptyline) has been investigated both in animal models and human patients (Di Rosso et al., 2016; Grosse et al., 2016; Himmerich et al., 2010; Alcocer-Gómez et al., 2017). However, a comparative assessment across a wider set of antidepressants is currently lacking.

In the present research, we aimed to address this knowledge gap by assessing the immunomodulatory potential of 21 different antidepressants at the transcriptomic level. To do so, we analysed transcriptomic signature data retrieved by the L1000 Connectivity Map (cMAP) database (https://clue.io/). This database is an ambitious project led by the Broad Institute that contains "1.5M gene expression profiles from 5,000 small-molecule compounds, and 3,000 genetic reagents, tested in multiple human cell types". This is the only dataset, to our knowledge, that allows the comparison of transcriptomic data for different compounds tested on the same set of cell lines, at the same experimental conditions, including exposure concentrations and quantification time points. Using this resource, we could explore the intricate relationships between drugs and specific genes of interest, which we refer to throughout this thesis as drug-gene interactions. In this context, drug-gene interactions explore how each antidepressant drug affects gene expression profiles, providing insights into their potential immunomodulatory effects.

Table 3.1 – Potency of Antidepressants for Blocking Some Neurotransmitter Transporters and Receptors. Abbreviations: 5-HT = serotonin, DA = dopamine, NE = norepinephrine, SSRI = Selective serotonin reuptake inhibitors, SNRI = Serotonin-norepinephrine reuptake inhibitors, SARI = Serotonin antagonists and reuptake inhibitors, NDRI = Norepinephrine-dopamine reuptake inhibitors, TCA = Tricyclic antidepressants, TeCA = Tetracyclic antidepressants. Symbols: + to ++++++ = increasing levels of potency, - = weak, 0 = no effect. Adapted from (Richelson, 2003).

Drug	Class	Neutrotransmitter Transporters			Receptors		
		NE	5-HT	DA	Histamine H1	Muscarinic Acetylcholine	α1-Adrenergic
Amitriptyline	TCA	+++	++++	-	+++++	+++	+++
Amoxapine	TCA	+++	+++	-	+++	+	+++
Bupropion	NDRI	0	-	+	-	0	-
Citalopram	SSRI	-	+++++	0	+	-	+
Clomipramine	TCA	+++	++++++	-	+++	+++	+++
Desipramine	TCA	+++++	+++	-	++	++	++
Doxepin	TCA	+++	+++	0	++++++	++	+++
Duloxetine	SNRI	++++	++++++	+	-	-	-
Fluoxetine	SSRI	++	+++++	-	-	-	-
Fluvoxamine	SSRI	+	+++++	-	0	0	-
Imipramine	TCA	+++	+++++	-	++++	++	++
Mirtazapine	TeCA	-	0	0	++++++	+	+
Nefazodone	SARI	++	++	++	+++	-	+++
Paroxetine	SSRI	+++	++++++	+	0	++	-
Sertraline	SSRI	+	++++++	+++	0	+	++
Venlafaxine	SNRI	+	++++	-	0	0	0

Table 3.2 – Adverse effects of antidepressant drugs, based on mechanism of action. Adapted from (Richelson, 2003; Khawam et al., 2006).

Property	Possible adverse effect
Norepinephrine transporter blockade	Anxiety Augmentation of pressor effects of sympathomimetic amines Diaphoresis Tachycardia Tremor
Serotonin reuptake inhibition	Anorexia early in the treatment and weight gain later Dose-dependent increase or decrease in anxiety Ejaculatory disturbances, anorgasmia, and decreased libido Extrapyramidal side effects Interaction with mono-amine oxidase inhibitors and tryptophan Nausea, vomiting, and diarrhea. Sedation or insomnia Serotonin syndrome
Dopamine reuptake inhibition	Activation and aggravation of psychosis Parkinsonism Psychomotor activation
Alpha-1 adrenergic receptor blockade	Postural hypotension and dizziness Potentiation of the antihypertensive effect of other medications Reflex tachycardia
Dopamine D2 receptor blockade	Extrapyramidal side effects: akathisia, dystonia, parkinsonism, tardive dyskinesia Endocrine effects; prolactin elevation
Histamine H1 receptor blockade	Drowsiness Falls in the elderly Orthostatic hypotension Sedation Weight gain
Muscarinic acetylcholine receptor blockade	Blurred vision Central effects: memory and cognitive impairment, delirium in severe cases Gastrointestinal effects: decreased salivation, dry mouth, decreased peristalsis, constipation Precipitation of narrow-angle glaucoma Sinus tachycardia Urinary hesitancy and retention

3.3 Methods

3.3.1 Pathway enrichment of drug-gene interactions using CTD and Reactome

The first approach used to assess the ability of a selected set of antidepressants to modulate immune and inflammation-related pathways was to evaluate the functional value of drug-gene interaction data extracted from the comparative Toxicogenomics Database (CTD). The CTD is a publicly available database that provides manually curated information about chemical–gene, chemical–disease, and gene–disease relationships (Davis et al., 2018). Drug-gene interaction data extracted from the CTD was then used to assess the ability of the compounds of interest to perturb relevant biological pathways hosted in the Reactome Knowledgebase (https://reactome.org) (Fabregat et al., 2018). Specifically, the analysis was focused on Reactome biological pathways related to the immune system: cytokine signalling in the immune system (Table 3.3), innate immune system (Table 3.4), and adaptive immune system (Table 3.5). For the purpose of this analysis, these were considered pathways of interest.

At this stage, the two most prevalent classes of antidepressants were investigated: SSRIs and TCAs. A list of antidepressants was compiled from the literature (Wishart et al., 2018), and drug selection was conducted taking the following factors into consideration: oral administration route and approved status in the USA (FDA, 2018), the UK (MHRA, 2018), and Europe (European Medicines Agency, 2018). The drug-gene interaction data extracted from CTD (Davis et al., 2018) was filtered to exclude any drug that presented less than 10 drug-gene interactions in the database. To assess the reproducibility of drug-gene interaction data across drugs within the same class, an intra-class frequency analysis was performed and genes interacting only with one drug were excluded. A list of the antidepressants studied and their respective number of drug-gene interactions available on CTD can be found in Table 3.6.

At this stage, the two most prevalent classes of antidepressants were investigated: SSRIs and TCAs. A list of antidepressants was compiled from literature (Wishart et al., 2018), and drug selection was conducted taking the following factors into consideration: the drug is administered orally; the drug has verified approval status in the USA

(FDA, 2018), the UK (MHRA, 2018), and Europe (European Medicines Agency, 2018). The drug-gene interaction data extracted from CTD (Davis et al., 2018) was filtered to exclude any drug that presented under 10 drug-gene interactions in the database. An intra-class frequency analysis was performed on all remaining drug-gene interactions, and genes interacting only with one drug were excluded. The resulting genes were ranked by the number of drug-gene interactions with the chosen drugs, allowing for the identification of target genes more commonly shared among drugs within each class. A list of the antidepressants studied, and their respective number of drug-gene interactions available on CTD can be found in Table 3.6.

Pathway enrichment for the drug-gene interactions of each drug was then performed in Reactome (Fabregat et al., 2018). Pathways linked to the innate immune system and cytokine signalling for each drug were extracted along with their false discovery rate (FDR). Based on these values, heat-map was created and can be found in Section 3.4.1. Due to limitations arising from data heterogeneity in the information extracted from CTD (as described in Section 3.4.1), an alternative data source for drug-gene interactions was selected and is described in Section 3.3.2 below.

Table 3.3 – Biological pathways related to cytokine signalling in the immune system as described in the Reactome Pathway Browser.

Interferon signalling	Interferon alpha/beta signalling
	Interferon gamma signalling
	Antiviral mechanism by IFN-stimulated genes
Signalling by interleukins	Interleukin-1 family signalling
	Interleukin-2 family signalling
	Interleukin-3, 5 and GM-CSF signalling
	Interleukin-4 and 13 signalling
	Interleukin-6 family signalling
	Interleukin-7 signalling
	Interleukin-10 signalling
	Interleukin-12 family signalling
	Interleukin-17 signalling
	Interleukin-20 family signalling
	Other interleukin signalling
Growth hormone receptor signalling	
Prolactin receptor signalling	
TNFR2 non-canonical NF-kB pathway	

Table 3.4 – Biological pathways related to the innate immune system as described in the Reactome pathway browser.

Toll-like Receptor Cascades	Trafficking and processing of endosomal TLR Toll Like Receptor 10 (TLR10) Cascade Toll Like Receptor 3 (TLR3) Cascade Toll Like Receptor 5 (TLRS) Cascade Toll Like Receptor 7/8 (TLR7/8) Cascade Toll Like Receptor 9 (TLR9) Cascade Toll Like Receptor 4 (TLR4) Cascade Toll Like Receptor 2 (TLR2) Cascade Regulation of TLR by endogenous ligand
Complement cascade	Initial triggering of complement Activation of C3 and C5 Terminal pathway of complement Regulation of Complement cascade
Nucleotide-binding domain leucine-rich repeat containing receptor (NLR) signalling pathways	NOD1/2 signalling Pathway Inflammasomes
DDX58/IFIH1-mediated induction of interferon-alpha/beta	TRAF3-dependent IRF activation pathway NF-kB activation through FADD/RIP-1 pathway mediated by caspase-8 and -10 TRAF6 mediated IRF7 activation TRAF6 mediated NF-kB activation Negative regulators of DDX58/IFIH1 signalling
Cytosolic sensors of pathogen-associated DNA	ZBP1(DAI) mediated induction of type I IFNs STING mediated induction of host immune responses LRR FLIT-interacting protein 1 (LRRFIP1) activates type I IFN production DEx/H-box helicases activate type I IFN and inflammatory cytokines production Regulation of innate immune responses to cytosolic DNA
Fcgamma receptor (FCGR) dependent phagocytosis	FCGR activation Regulation of actin dynamics for phagocytic cup formation Role of phospholipids in phagocytosis
DAP12 interactions	
Fc epsilon receptor (FCERI) signalling	

Table 3.4 – Biological pathways related to the innate immune system as described in the Reactome pathway browser (*Continued*).

C-type lectin receptors (CLRs)	CLEC7A (Dectin-1) signalling Dectin-2 family CD209 (DC-SIGN) signalling
Antimicrobial peptides	Defensins Metal sequestration by antimicrobial proteins Ion influx/efflux at host-pathogen interface
Advanced glycosylation endproduct receptor signalling	
Neutrophil degranulation	
ROS and RNS production in phagocytes	
Alpha-protein kinase 1 signalling pathway	

Table 3.5 – Biological pathways related to the adaptive immune system as described in the Reactome Pathway Browser.

TCR signalling	Phosphorylation of CD3 and TCR zeta chains Translocation of ZAP-70 to Immunological synapse Generation of second messenger molecules Downstream TCR signalling
Costimulation by the CD28 family	CD28 co-stimulation CTLA4 inhibitory signalling PD-1 signalling
Signalling by the B Cell Receptor (BCR)	Antigen activates B Cell Receptor (BCR) leading to generation of second messengers Downstream signalling events of B Cell Receptor (BCR) CD22 mediated BCR regulation
Class I MI-IC mediated antigen processing & presentation	Antigen processing: Ubiquitination & Proteasome degradation Antigen Presentation: Folding, assembly and peptide loading of class I MC Antigen processing-Cross presentation
MHC class II antigen presentation	
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	
Rap1 signalling	
Butyrophilin (BTN) family interactions	

Table 3.6 – List of antidepressants studied and their respective number of drug-gene interactions available on CTD. SSRI = Selective serotonin reuptake inhibitors, TCA = Tricyclic antidepressants.

Drug name	Class	Drug-gene interactions
Fluoxetine	SSRI	362
Sertraline	SSRI	118
Citalopram	SSRI	43
Paroxetine	SSRI	43
Fluvoxamine	SSRI	22
Amitriptyline	TCA	196
Imipramine	TCA	124
Clomipramine	TCA	95
Nortriptyline	TCA	22
Doxepin	TCA	17

3.3.2 Functional enrichment analysis using LINCS L1000 and Reactome

To overcome the heterogeneity of the data sources associated with the CTD database, the second approach used in this study involved a more homogeneous set of experimental data generated using the same platform and study design for all drugs of interest. Specifically, drug-specific data was extracted from the cMAP, a publicly available database which has collected gene expression profiles for thousands of perturbagens at a variety of time points, doses, and cell lines (Subramanian et al., 2017). The transcriptomic data available in this cMAP was obtained through the L1000 assay, a high-throughput gene-expression profiling assay based on the direct measurement of a reduced representation of the transcriptome and subsequent computational inference of the portion of the transcriptome not explicitly measured. The L1000 assay contains 1,058 probes for 978 landmark transcripts and 80 control transcripts. It has been demonstrated to be highly reproducible, comparable to RNA sequencing, and suitable for computational inference of the expression levels of 81% of non-measured transcripts, equating to 11,350 additional genes (Subramanian et al., 2017; Duan et al., 2014). The LINCS L1000 data repository contains almost two million gene expression profiles for thousands of small molecules and drugs. Data is separated into five data levels at different points in the analysis pipeline (Table 3.7).

Differential gene expression signatures for twenty antidepressants and one anti-inflammatory drug were extracted from the level 5 database. Antidepressants across all major classes and available on the platform were selected at this stage (Table 3.8). The antidepressants studied have also been demonstrated to be more effective than placebo in clinical trials (Cipriani et al., 2018). To allow for comparison, data for the glucocorticoid anti-inflammatory drug dexamethasone was also extracted. Dexamethasone data was analysed separately, following the same methods described for antidepressants.

Table 3.7 – Levels of the data processing pipeline of the LINCS L1000 data set (Subramanian et al., 2017).

Level 1	Raw unprocessed flow cytometry data from Luminex (LXB)
Level 2	Gene expression values per 1000 genes after deconvolution (GEX)
Level 3	Quantile-normalized gene expression profiles of landmark genes and imputed transcripts (Q2NORM or INF)
Level 4	Gene signatures computed using z-scores relative to the plate population as control (ZSPCINF) or relative to the plate vehicle control (ZSVCINF)
Level 5	Differential gene expression signatures

The dataset extracted for the selected 20 antidepressants contained a total of 756 samples relevant to the study. As the majority of the samples (744) were collected from cultures exposed to 10µM of drug, in order to keep the data consistent, the remaining 12 samples (4 each for 0.15625, 0.625 and 2.5 µM) were removed from the data set. The remaining data set contained samples from 16 cell lines (Table 3.9) and 2 time points - 6h and 24h. The full set of genes extracted for all drugs, cell lines and time points (12331) was used for further analysis.

In order to obtain representative gene expression profiles, the samples were handled in two ways: one dataset was produced by considering the gene expression profiles per drug and timepoint, calculated by averaging the data obtained with all cell lines; the second dataset was obtained by considering the results obtained for each individual cell-line/drug/time point combination Using these methods, the first dataset contained a total of 40 gene expression profiles - one per time point for each of the twenty antidepressants - while the second dataset contained a total of 372 gene ex-

Table 3.8 – List of antidepressants investigated using the LINCS L1000 data repository and their respective antidepressant class. TCA = Tricyclic antidepressants; SSRI = Selective serotonin reuptake inhibitors; SNRI = Serotonin–norepinephrine reuptake inhibitors; SARI = Serotonin antagonists and reuptake inhibitors; NRI = Norepinephrine reuptake inhibitors; NDRI = Norepinephrine–dopamine reuptake inhibitors; MAOI = mono-amine oxidase inhibitors

Name	Class
Amitriptyline	TCA
Clomipramine	TCA
Dosulepin	TCA
Doxepin	TCA
Nortriptyline	TCA
Citalopram	SSRI
Escitalopram	SSRI
Fluoxetine	SSRI
Fluvoxamine	SSRI
Sertraline	SSRI
Paroxetine	SSRI
Duloxetine	SNRI
Milnacipran	SNRI
Venlafaxine	SNRI
Nefazodone	SARI
Trazodone	SARI
Reboxetine	NRI
Bupropion	NDRI
Moclebamide	MAOI

pression profiles - one for each combination of drug, time point and cell line available.

The data from the first set containing 40 drug-induced gene expression profiles, one for each drug and time point, were analysed using QIAGEN ingenuity Pathway Analysis (IPA) (Krämer et al., 2014). However, because the IPA software limits analyses to 20 samples at a time, the enrichment analysis of the gene expression profile dataset that included all combinations of drug, time point, and cell-line (372 profiles), was instead conducted using Reactome. As in section 3.3.1, the biological pathways related to the immune system listed in Reactome - cytokine signalling in immune system (Table 3.3), innate immune system (Table 3.4), and adaptive immune system (Table 3.5) - were selected as pathways of interest. To visualize the number of differential expressed genes associated with pathways of interest and respective p-adjusted values, dot plots were produced. The gene ratio (number of significant genes associated with the pathways / total number of significant genes) is indicated by a colour gradient, while

Table 3.9 – Cell lines used to quantify the transcriptomic signature of the selected antidepressants. (De Wolf et al., 2016).

Cell line	Organ	Description
A375	Skin	Malignant melanoma cell line
A549	Lung	Lung Carcinoma cell line
ASC	Adipose	Adipose stromal cell
FIBRNPC	Stem-cell	Induced pluripotent stem cell
HA1E	Kidney	Immortalized kidney cell line
HCC515	Lung	Lung carcinoma cell line
HEK293T	Kidney	Immortalized human embryonic kidney cells
HEPG2	Liver	Hepatocellular carcinoma cell line
HT29	Colon	Colon adenocarcinoma cell line
MCF7	Breast	Breast adenocarcinoma cell line
NEU	Brain	Primary terminally differentiated neuron cells
NPC	Brain	Primary iPS-derived neural progenitor cells
PC3	Prostate	Prostate adenocarcinoma cell line
PHH	Liver	Primary hepatocyte cells
SKB	Muscle	Skeletal myoblast cells
VCAP	Prostate	Metastatic prostate cancer cell line

the enrichment scores (adjusted p-values) are encoded as dot size.

Data was analysed using the following R packages: ReactomePA (Yu and He, 2016), reactome.db (Ligtenberg, 2019), clusterProfiler (Wu et al., 2021), ggplot2 (Wickham, 2016), reshape2 (Wickham, 2007), and viridis (Garnier et al., 2022). The summarized results from this enrichment analysis can be visualised in a dedicated Shiny (Chang et al., 2023) web app (http://pgb.liv.ac.uk/shiny/agraus/Inflammation) and are presented in Section 3.4.2.

3.4 Results

3.4.1 Pathway enrichment of drug-gene interactions using CTD and Reactome

Initially, the pathway enrichment analysis was conducted by examining drug-gene interactions for each drug using the software Gene Analytics (Ben-Ari Fuchs et al., 2016). However, it became apparent early on that Gene Analytics had a major limitation: it couldn't categorize pathways into classes, which made it impossible to select pathways related to immunity and inflammation. To overcome this, a new pathway enrichment

was performed using Reactome.

However, the results obtained from Reactome using data from CTD were not as comprehensive as anticipated. During data extraction, it became clear that the CTD database had a significant limitation in terms of data gaps, as shown in Figures 3.1 and 3.2. The data available on CTD covers different species, cell types, experiments, methods, and concentrations, making it challenging to compare results. Additionally, the data is restricted to target genes that the experiments focused on, providing a skewed view of the drug-gene interaction. As a result, a new, highly standardized data source was procured to allow for proper comparison of drug-gene interaction results. Therefore, the methods using CTD and Gene Analytics were not further explored and efforts were focused on the method using LINCS 1000 data enriched using Reactome. The tables containing the Reactome pathway enrichment results can be found in Appendix E.

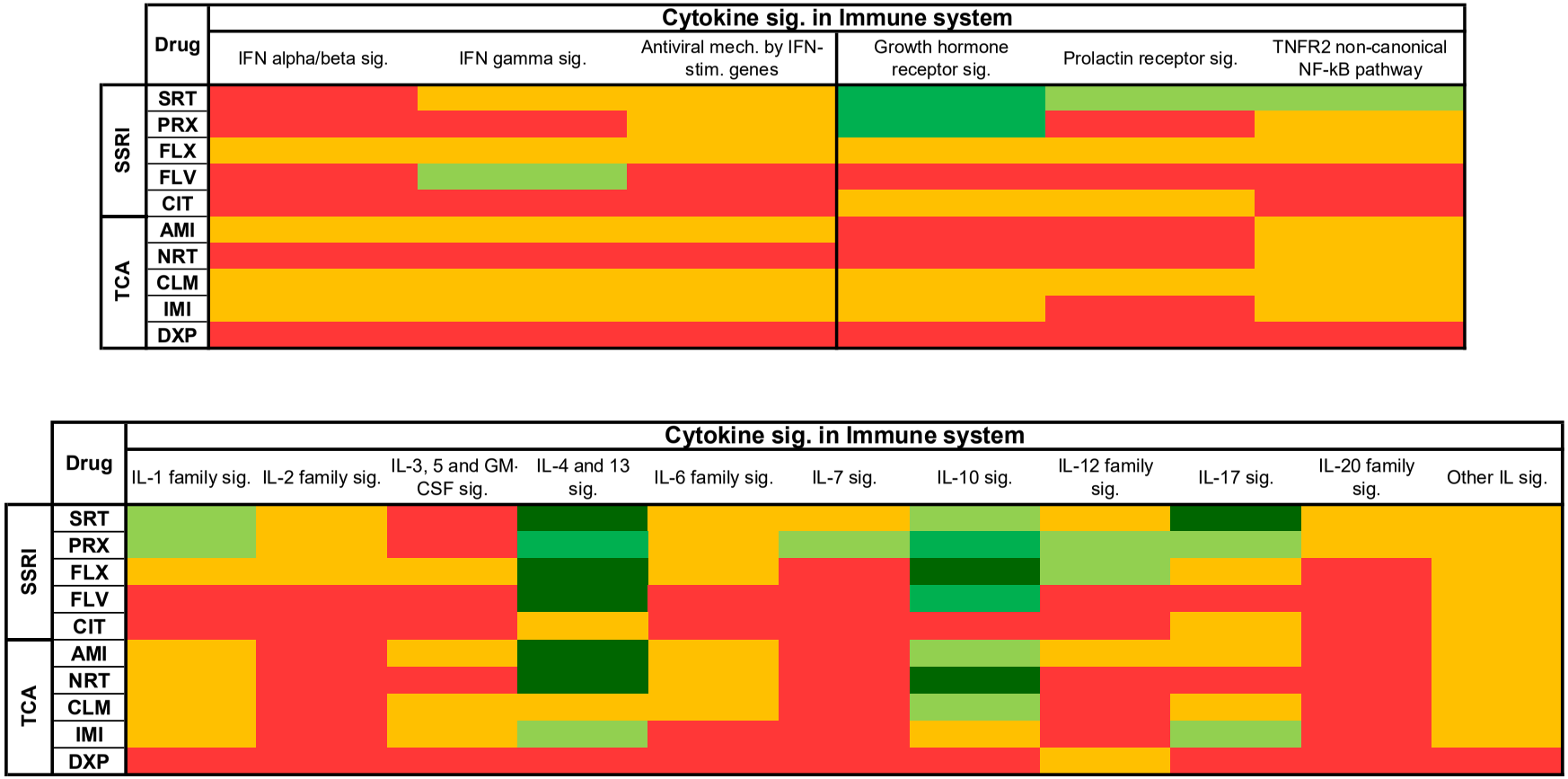

Figure 3.1 – Enrichment results for pathways related to cytokine signalling in the immune system for five SSRI and five TCA antidepressants. Red - no data; orange - FDR >0.05; light green - FDR <= 0.05; green - FDR <= 0.005; dark green - FDR <= 0.0005. SRT = sertraline; PRX = paroxetine; FLX = fluoxetine; FLV = fluvoxamine; CIT = citalopram; AMI = amitriptyline; NRT = nortriptylene; CLM = clomipramine; IMI = imipramine; DXP = doxepin; SSRI = Selective serotonin reuptake inhibitors; TCA = Tricyclic antidepressants.

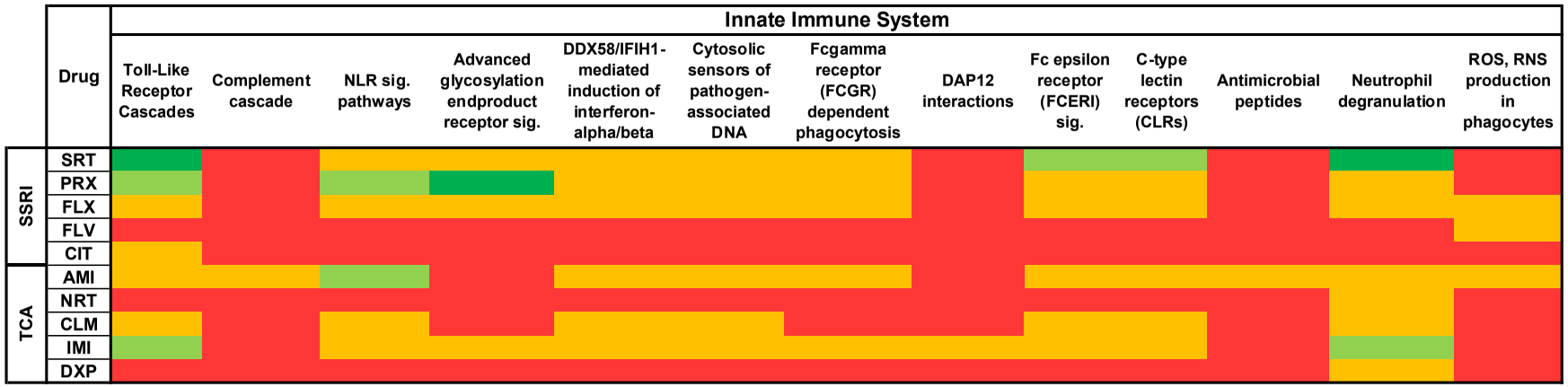

Figure 3.2 – Enrichment results for pathways related to the innate immune system for five SSRI and five TCA antidepressants. Red - no data; orange - FDR >0.05; light green - FDR <= 0.05; green - FDR <= 0.005; dark green - FDR <= 0.0005. SRT = sertraline; PRX = paroxetine; FLX = fluoxetine; FLV = fluvoxamine; CIT = citalopram; AMI = amitriptyline; NRT = nortriptylene; CLM = clomipramine; IMI = imipramine; DXP = doxepin; SSRI = Selective serotonin reuptake inhibitors; TCA = Tricyclic antidepressants.

3.4.2 Functional enrichment analysis using LINCS L1000 and Reactome

The results from the first data set of 40 profiles, analysed using Ingenuity Pathway Analysis, did not reveal any significant differences between compounds and mainly indicated a connection to cancer-related processes. This can be attributed to the fact that most of the cell lines used in the study were cancer cells (Table 3.9). By averaging the gene expression profiles across cell lines, it is likely that some of the unique differences between cell lines were disregarded, as different cell lines are expected to display varying responses to the same drug. By averaging the data, differences among the cell lines were reduced, potentially highlighting common features such as cancer-related pathways.

The second data set, which consisted of 372 gene expression profiles, one for each combination of drug, time point and cell line available, was more detailed.

The number of total enriched pathways for each antidepressant, cell-line, and time-point can be found in Figure 3.3, while the pathways of interest per antidepressant are presented in Table 3.10. The majority of antidepressants studied interacted with multiple pathways, with some drugs interacting with up to 17 pathways. Of the 20 antidepressants analysed, only two, doxepin and mirtazapine, did not interact with any pathways of interest, while 7 interacted with only 1 or 2 pathways of interest. The results for each antidepressant and anti-inflammatory dexamethasone, can be found in Appendix F.

As expected, different lines displayed varying responses to the same drug. Generally, the response to an antidepressant at each time point was different. In the majority of cases, pathway interactions at different time points were observed in different cell lines (Table 3.14). Equally, the same pathways did not seem to be exclusively activated in certain cell lines, with each antidepressant presenting results for 1 to 8 cell lines for a single target (Table 3.15). While cell lines HCC515 (lung carcinoma cell line) and A375 (malignant melanoma cell line), interacted with the highest number of antidepressants, HA1E (immortalized kidney cell line) and HEPG2 (hepatocellular carcinoma cell line) had the most immune pathways interactions across all drugs.

Pathway interactions in the immune system were classified in 3 groups, according to Reactome: cytokine signalling in the immune system, innate immune system and adaptive immune system.

Cytokine signalling had the most pathway interactions among the three groups, with 50% of antidepressants showing interaction with IFN alpha and beta signalling and 30% with Tumor necrosis factor receptor type II (TNFR2) non-canonical nuclear factor kappa B (NF-κB) pathway (Table 3.11). No particular antidepressant class demonstrated a higher level of interaction with cytokine signalling pathways, as SARIs, TCAs, and SSRIs interacted with multiple pathways.

While both dosulepin and nortriptylene, TCAs, interacted with multiple cytokine signalling pathways, amitriptyline and doxepin, also TCAs, showed low to no interactions. Similarly, SSRIs displayed a varied response with sertraline having the highest number of interactions, and citalopram, escitalopram, and fluvoxamine having none. Among the SARIs, trazodone showed the most interactions in the cytokine signalling group (9), while nefazodone only affected 3 pathways.

While SSRIs had mixed results in the cytokine signalling group, they had limited interactions in the innate immune system (Table 3.12). TCAs also showed a varied response, with dosulepin interacting with 5 pathways and amitriptyline with only one. As with cytokine signalling, trazodone had the highest number of interactions in the innate immune system. The most active pathways across all antidepressants included tumor necrosis factor receptor-associated factor 6 (TRAF6)-mediated interferon regulatory factor (IRF)7 activation, neutrophil degranulation, and high-affinity IgE receptor (FCERI) signalling, as well as some activity in TLR 2 and 3 cascades.

Despite low interaction in the cytokine signalling and innate immune system groups, amitriptyline was among the most active drugs in the adaptive immune system (Table 3.13). TCAs, in general, were highly active in this group, with dosulepin ranking second. SARIs displayed high pathway interaction in the adaptive immune system, while SSRIs displayed mixed results, with sertraline interacting with 5 pathways but several other SSRIs having no interactions. The most active pathways in the adaptive immune system group were antigen processing-Cross presentation and downstream

TCR signalling.

Overall, among all the antidepressants, trazodone had the highest number of pathway interactions, followed by dosulepin. The pathways linked to each antidepressant showed a wide diversity of targets not only between classes but also within the same class. Looking at the two biggest classes SSRIs and TCAs as an example, it becomes evident that even within the same class the immunomodulatory potential of each antidepressant is varied, with antidepressants exerting their action through different pathways (Table 3.16).

Meanwhile, dexamethasone triggered the expected immune pathways involving TLR cascades, IL signalling, NF-κB activation. Notably, all but one (IL-12 family signalling) of the pathways activated by dexamethasone, were activated by different antidepressants.

Table 3.10 – Number of inflammation-related pathways of interest for each antidepressant at 2-time points, 6 and 24 hours of exposure.

Drug	6h	24h
Amitriptyline	1	8
Bupropion	0	2
Citalopram	2	0
Clomipramine	0	1
Dosulepin	15	7
Doxepin	0	0
Duloxetine	0	1
Escitalopram	0	1
Fluoxetine	1	1
Fluvoxamine	1	0
Milnacipran	5	0
Mirtazapine	0	0
Moclobemide	8	2
Nefazodone	8	2
Nortriptyline	2	7
Paroxetine	2	8
Reboxetine	11	2
Sertraline	1	11
Trazodone	17	12
Venlafaxine	4	2

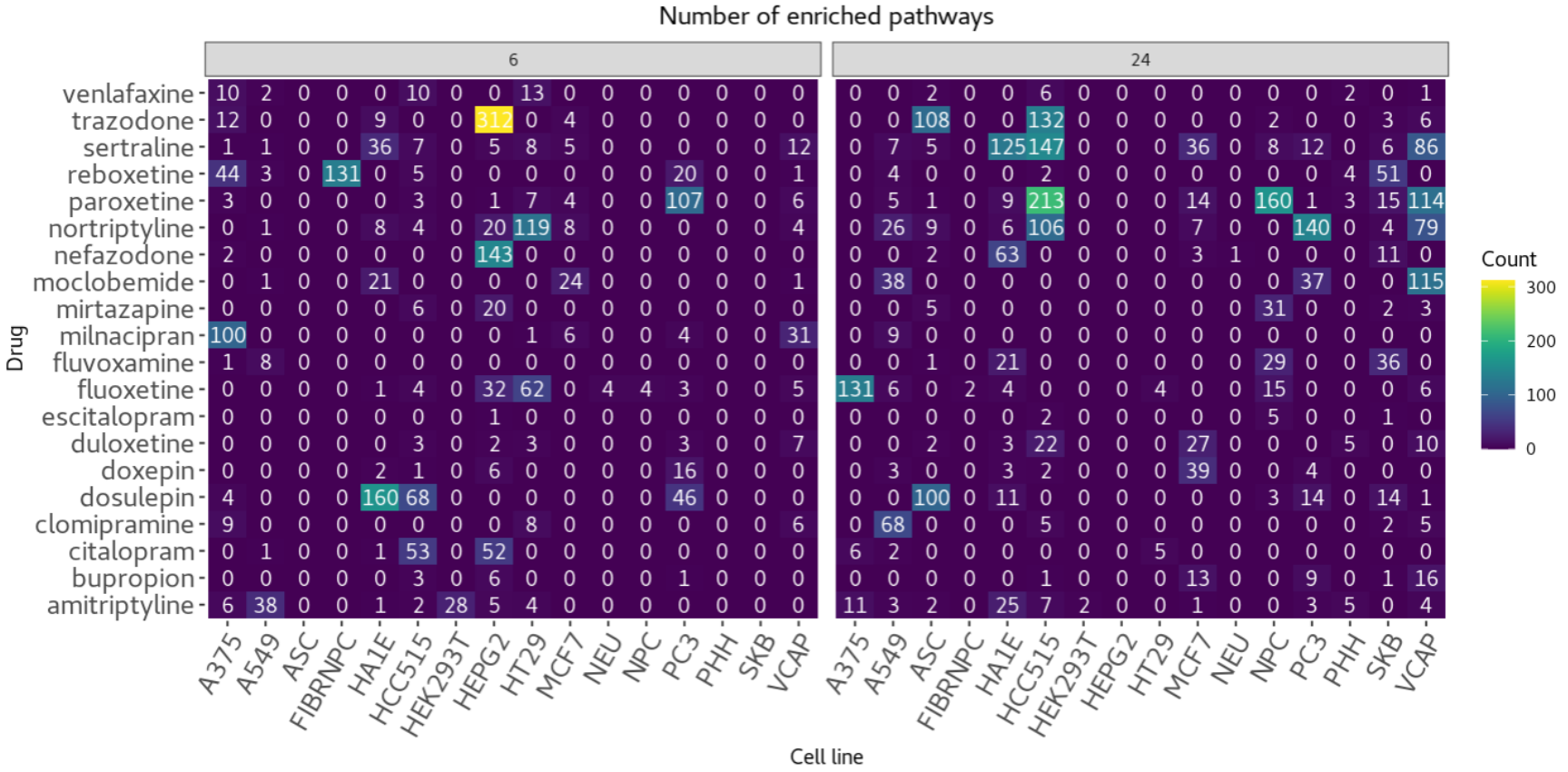

Figure 3.3 – Number of pathways enriched for each antidepressant at 2-time points, 6 and 24 hours of exposure.

Table 3.11 – Enrichment results for pathways related to cytokine signalling in the immune system for each drug. Only pathways linked to differentially expressed genes are displayed. 6 and 24 represent the exposure duration that elicited the effect. Freq. is the number of antidepressants affecting each pathway. DEX = dexamethasone; TRZ = trazodone; DSL = dosulepine; SRT = sertraline; NRT = nortriptylene; RBX = reboxetine; NFZ = nefazodone; PRX = paroxetine; FLX = fluoxetine; MCL = moclebemide; VEN = venlafaxine; AMI = amitriptyline; MIL = milnacipran; CLM = clomipramine; DLX = duloxetine; GC = glucocorticoid anti-inflammatory; SSRI = Selective serotonin reuptake inhibitors; SNRI = Serotonin–norepinephrine reuptake inhibitors; SARI = Serotonin antagonists and reuptake inhibitors; NRI = Norepinephrine reuptake inhibitors; TCA = Tricyclic antidepressants

Drug	**DEX**	**TRZ**	**DSL**	**SRT**	**NRT**	**RBX**	**NFZ**	**PRX**	**FLX**	**MCL**	**VEN**	**AMI**	**MIL**	**CLM**	**DLX**	**Freq.**
Drug class	GC	SARI	TCA	SSRI	TCA	NRI	SARI	SSRI	SSRI	MAO	SNRI	TCA	SNRI	TCA	SNRI	
Interferon alpha/beta signalling		6	6	24		24		24	6	24	6/24	6/24			24	10
TNFR2 non-canonical NF-kB pathway	6	6/24	6	24	6/24	6	6									6
Antiviral mechanism by IFN-stimulated genes		6	24	24				24	24							5
Interleukin-20 family signalling	6		6			6	6						6			4
Interleukin-1 family signalling	24	6/24	6	24			6									4
Interleukin-4 and 13 signalling	6/24	6	6/24	24	24											4
Interleukin-3, 5 and GM-CSF signalling	6	6/24	6					6/24								3
Interferon gamma signalling	6	24									6			24		3
Interleukin-17 signalling		6/24								6						2
Interleukin-10 signalling	24				24											1
Other interleukin signalling					24											1
Growth hormone receptor signalling						6										1
Prolactin receptor signalling			6													1
Interleukin-6 family signalling		24														1
Pathways affected by drug	8	9	8	5	4	4	3	3	2	2	2	1	1	1	1	

Table 3.12 – Enrichment results for pathways related to the innate immune system for each drug. Only pathways linked to differentially expressed genes are displayed. 6 and 24 represent the exposure duration that elicited the effect. Freq. is the number of antidepressants affecting each pathway. DEX = dexamethasone; TRZ = trazodone; DSL = dosulepine; RBX = reboxetine; MCL = moclebemide; NFZ = nefazodone; SRT = sertraline; AMI = amitriptyline; PRX = paroxetine; MIL = milnacipran; VEN = venlafaxine; BPR = bupropion; CIT = citalopram; ESC = escitalopram; FLV = fluvoxamine; GC = glucocorticoid anti-inflammatory; SARI = Serotonin antagonists and reuptake inhibitors; TCA = Tricyclic antidepressants; NRI = Norepinephrine reuptake inhibitors; MAO = mono-amine oxidase inhibitor; SSRI = Selective serotonin reuptake inhibitors; SNRI = Serotonin–norepinephrine reuptake inhibitors

Drug	**DEX**	**TRZ**	**DSL**	**RBX**	**MCL**	**NFZ**	**SRT**	**AMI**	**PRX**	**MIL**	**VEN**	**BPR**	**CIT**	**ESC**	**FLV**	**Freq.**
Drug class	GC	SARI	TCA	NRI	MAO	SARI	SSRI	TCA	SSRI	SNRI	SNRI	NDRI	SSRI	SSRI	SSRI	
TRAF6 mediated IRF7 activation		6			24	24		24			6/24		6	24	6	8
Neutrophil degranulation			24			6	24		6/24	6		24				6
Fc epsilon receptor (FCERI) signalling	6	6/24	6/24	6		6	6									5
Toll Like Receptor 3 (TLR3) Cascade	6	6		6/24	6											3
Toll Like Receptor 2 (TLR2) Cascade	6	6		2	6											3
Regulation of Complement cascade		24	24	6												3
Negative regulators of DDX58/IFIH1 signalling		6	24													2
Regulation of TLR by endogenous ligand			6													1
NOD1/2 signalling Pathway		6														1
Total genes hit	3	8	5	4	3	3	2	1	1	1	1	1	1	1	1	

Table 3.13 – Enrichment results for pathways related to the adaptive immune system for each drug. Only pathways linked to differentially expressed genes are displayed. 6 and 24 represent the exposure duration that elicited the effect. Freq. is the number of antidepressants affecting each pathway. DEX = dexamethasone; AMI = amitriptyline; DSL = dosulepine; SRT = sertraline; MCL = moclebemide; TRZ = trazodone; NFZ = nefazodone; PRX = paroxetine; RBX = reboxetine; NRT = nortriptylene; MIL = milnacipran; VEN = venlafaxine; BPR = bupropion; CIT = citalopram; GC = glucocorticoid anti-inflammatory; TCA = Tricyclic antidepressants; SSRI = Selective serotonin reuptake inhibitors; MAO = mono-amine oxidase inhibitor; SARI = Serotonin antagonists and reuptake inhibitors; NRI = Norepinephrine reuptake inhibitors; SNRI = Serotonin–norepinephrine reuptake inhibitors

Drug	**DEX**	**AMI**	**DSL**	**SER**	**MCL**	**TRZ**	**NFZ**	**PRX**	**RBX**	**NRT**	**MIL**	**VEN**	**BPR**	**CIT**	**Freq.**
Drug class	GC	TCA	TCA	SSRI	MAO	SARI	SARI	SSRI	NRI	TCA	SNRI	SNRI	NDRI	SSRI	
Antigen processing-Cross presentation	6	24	6	24		6/24	6	24	6	24	6	6		6	11
Downstream TCR signalling	6		6	24		6/24	6	24	6	24	6				8
Downstream signalling events of B Cell Receptor (BCR)	6		6	24		6/24	6	24	6	6/24	6				8
Generation of second messenger molecules		24	6/24		6	24									4
MHC class II antigen presentation				24	6			24					24		4
Phosphorylation of CD3 and TCR zeta chains		24	6		6										3
Translocation of ZAP-70 to Immunological synapse		24	6		6										3
Costimulation by the CD28 family		24			6										3
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell		24					24					6			2
Antigen processing: Ubiquitination & Proteasome degradation						6			6						2
Rap1 signalling				24											1
Total genes hit	3	6	6	5	5	5	4	4	4	3	3	2	1	1	

Table 3.14 – Cell-lines with active pathways per each drug. 6 and 24 represent the exposure duration that elicited the effect. DEX = dexamethasone; PRX = paroxetine; RBX = reboxetine; TRZ = trazodone; AMI = amitriptyline; DSL = dosulepine; MCL = moclebemide; NRT = nortriptylene; SRT = sertraline; BPR = bupropion; CIT = citalopram; FLX = fluoxetine; MIL = milnacipran; NFZ = nefazodone; CLM = clomipramine; DLX = duloxetine; ESC = escitalopram; FLV = fluvoxamine; VEN = venlafaxine.

Drug	# pathways	HCC515	A375	HA1E	PC3	HEPG2	SKB	A549	ASC	MCF7	NPC	PHH	VCAP	FIBRNPC	HEK293T	HT29
DEX	14	6		6	24			6			6			6		
PRX	6	24	6	24	6/24						24					
RBX	6						24					24				
TRZ	6	24	6	6		6	24		24							
AMI	4	6	24		24							24			24	
DSL	4	6		6			24		24							
MCL	3			6	24					6						
NRT	3				24								24			6
SER	3	6/24		24									24			
BPR	2	24					24									
CIT	2	6				6										
FLX	2		6			6										
MIL	2		6							6						
NFZ	2			24		6										
CLM	1		24													
DLX	1		24													
ESC	1	24														
FLV	1							6								
VEN	1	6/24														

Table 3.15 – Cell-lines with active pathways per each drug. The first section are pathways related to cytokine signalling in the immune system, the second innate immune system pathways and the third pathways in the adaptive immune system. (6) and (24) represent the exposure duration that elicited the effect. DEX = dexamethasone; PRX = paroxetine; RBX = reboxetine; TRZ = trazodone; AMI = amitriptyline; DSL = dosulepine; MCL = moclebemide; NRT = nortriptylene; SRT = sertraline; BPR = bupropion; CIT = citalopram; FLX = fluoxetine; MIL = milnacipran; NFZ = nefazodone; CLM = clomipramine; DLX = duloxetine; ESC = escitalopram; FLV = fluvoxamine; VEN = venlafaxine.

	HA1E	HEPG2	HCC515	A375	ASC	PC3	MCF7	SKB	FIBRNPC	A549	NPC	PHH	VCAP	HT29
Interferon alpha/beta signalling	SRT (24)	FLX (6)	AMT (6) DSL (6) VEN (6)/(24)	TRZ (6)/(24)	DLX (24)	MCL (24) AMT (24)		PRX (24)				RBX (24)		
TNFR2 non-canonical NF-kB pathway	DSL (6) SRT (24)	NFZ (6) TRZ (6)			TRZ (24)	NRT (24)			RBX (6)					NRT (6)
Interleukin-20 family signalling	DSL (6)	NFZ (6)		MLN (6)	TRZ (24)				RBX (6)					
Antiviral mechanism by IFN-stimulated genes	SRT (24)	TRZ (6)	PRX (24)	FLX (24)	DSL (24)									
Interleukin-1 family signalling	DSL (6) SRT (24)	NFZ (6) TRZ (6)	TRZ (24)											
Interleukin-4 and 13 signalling	SRT (24)	TRZ (6)	DSL (6) PRX (24)					DSL (24)					NRT (24)	
Interleukin-3, 5 and GM-CSF signalling	DSL (6)	TRZ (6)			TRZ (24)	PRX (6)								
Interferon gamma signalling			VEN (6)		TRZ (24)					CLM (24)				
Interleukin-10 signalling													NRT (24)	
Interleukin-17 signalling	MCL (6)	TRZ (6)												
Other interleukin signalling						NRT (24)								
Growth hormone receptor signalling									RBX (6)					
Prolactin receptor signalling	DSL (6)													
Interleukin-6 family signalling					TRZ (24)									
TRAF6 mediated IRF7 activation	NFZ (24)	CIT (6)	ESC (24) VEN (24)	TRZ (6)		MCL (24)				FLV (6)				
Neutrophil degranulation	PRX (24) SRT (24)	NFZ (6)	BPR (24)	PRX (6)	DSL (24)		MLN (6)							
Fc epsilon receptor (FCERI) signalling	DSL (6)	NFZ (6) TRZ (6)	SRT (6)	RBX (6)	DSL (24) TRZ (24)									
Toll Like Receptor 3 (TLR3) Cascade	MCL (6)	TRZ (6)				RBX (6)		RBX (24)						
Toll Like Receptor 2 (TLR2) Cascade	MCL (6)	TRZ (6)				RBX (6)								
Regulation of Complement cascade					DSL (24) TRZ (24)					RBX (6)				
Negative regulators of DDX58/IFIH1 signalling		TRZ (6)			DSL (24)									
Regulation of TLR by endogenous ligand	DSL (6)													
NOD1/2 signalling Pathway		TRZ (6)												
Antigen processing-Cross presentation	DSL (6) SRT (24) TRZ (6)	NFZ (6) TRZ (6)	CIT (6) TRZ (24) VEN (6)	MLN (6)		NRT (24)			RBX (6)			AMT (24)		
Downstream TCR signalling	DSL (6) SRT (24)	NFZ (6) TRZ (6)	TRZ (24)	MLN (6) RBX (6)		NRT (24)					PRX (24)			
Downstream signalling events of B Cell Receptor (BCR)	DSL (6) SRT (24)	NFZ (6) TRZ (6)	TRZ (24)	MLN (6) RBX (6)		NRT (24)					PRX (24)			NRT (6)
Phosphorylation of CD3 and TCR zeta chains	DSL (6)			AMT (24)			MCL (6)							
Translocation of ZAP-70 to Immunological synapse	DSL (6)						MCL (6)							
Generation of second messenger molecules	DSL (6)			AMT (24)			MCL (6)	DSL (24) TRZ (24)						
MHC class II antigen presentation	NFZ (24)		PRX (24)	AMT (24)			MCL (6)	BPR (24)			PRX (24)		SRT (24)	
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell			VEN (6)									AMT (24)		
Costimulation by the CD28 family				AMT (24)			MCL (6)							
Antigen processing: Ubiquitination & Proteasome degradation		TRZ (6)				RBX (6)								

Table 3.16 – Comparison of results for SSRIs and TCAs as an example of the variability of the immunomodulatory potential of antidepressants within the same class.

Drug	DSL	NRT	AMI	CLM	SRT	PRX	FLX	CIT	ESC	FLV
Drug class	TCA	TCA	TCA	TCA	SSRI	SSRI	SSRI	SSRI	SSRI	SSRI
Interferon alpha/beta signalling	6		6/24		24	24	6			
TNFR2 non-canonical NF-kB pathway	6	6/24			24					
Interleukin-20 family signalling	6									
Antiviral mechanism by IFN-stimulated genes	24				24	24	24			
Interleukin-1 family signalling	6				24					
Interleukin-4 and 13 signalling	6/24	24			24					
Interleukin-3, 5 and GM-CSF signalling	6					6/24				
Interferon gamma signalling				24						
Interleukin-10 signalling		24								
Interleukin-17 signalling										
Other interleukin signalling		24								
Prolactin receptor signalling	6									
TRAF6 mediated IRF7 activation			24					6	24	6
Neutrophil degranulation	24				24	6/24				
Fc epsilon receptor (FCERI) signalling	6/24				6					
Regulation of Complement cascade	24									
Negative regulators of DDX58/IFIH1 signalling	24									
Regulation of TLR by endogenous ligand	6									
Antigen processing-Cross presentation	6	24	24		24	24		6		
Downstream TCR signalling	6	24			24	24				
Downstream signalling events of B Cell Receptor (BCR)	6	6/24			24	24				
Phosphorylation of CD3 and TCR zeta chains	6		24							
Translocation of ZAP-70 to Immunological synapse	6		24							
Generation of second messenger molecules	6/24		24							
MHC class II antigen presentation					24	24				
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell			24							
Costimulation by the CD28 family			24							
Rap1 signalling					24					

3.5 Discussion

3.5.1 Cytokine signalling in the immune system

In our enrichment analysis of the differential gene expression profiles, antidepressants showed high levels of interaction with pathways related to cytokine signalling in the immune system, including various IL signalling pathways

3.5.1.1 Interferons-α, -β and -γ

Our analysis found that 50% of the antidepressants investigated across all classes interact with the IFN-α and -β signalling pathway. This highlights the significance of IFNs, which play a crucial role in inflammation and immunoregulation. On the other hand, only three antidepressants, clomipramine, trazodone, and venlafaxine, were found to interact with the IFN-γ signalling pathway. This suggests that some components of the immune system may have varying reactions to different types of antidepressants.

IFNs stimulate both macrophages and NK cells to elicit an anti-viral and anti-tumor response, involving IRF 3 and IRF7 antiviral pathways. Type I IFNs, specifically IFN-α and IFN-β, are used to treat a number of medical conditions, including chronic hepatitis B and C virus infections (Raison et al., 2005; Li et al., 2018). Although they are effective in treating these conditions, they also induce significant depressive symptoms in patients (Raison et al., 2005; Coch et al., 2019), highlighting the complex relationship between the immune system and mental health. Recent studies have demonstrated that antidepressants are effective in treating IFN-induced depression. A meta-analysis of 8 clinical studies found that pre-treatment with selective SSRIs can lower the incidence and severity of depression associated with IFN therapy in patients with chronic hepatitis C infection or malignant melanoma (Sarkar and Schaefer, 2014).

The interaction between antidepressants and the IFN signalling pathway has also been linked in our analysis to the transcriptional activation of IFN-α, mediated by the IRF7 activation pathway. The TRAF6 mediated IRF7 activation pathway was linked to 40% of drugs investigated. IRF7 is linked to the transcriptional activation of IFN-α and has been identified as a gene of interest in another study investigating genomic factors associated with antidepressant response. In patients receiving citalopram treatment,

IRF7 was the most significantly differentially expressed gene and its expression was upregulated in individuals who responded to treatment (Mamdani et al., 2011).

IFN-γ, a type II IFN, is a crucial mediator of cell-mediated immunity and has diverse, mainly pro-inflammatory actions on immunocytes and target tissue (Bhat et al., 2017). IFN-γ induces indoleamine 2,3-dioxygenase (IDO), which catalyzes the conversion of tryptophan to kynurenine in the kynurenine pathway. This reaction leads to decreased serotonin levels and overactivity of the glutamatergic system, both of which have been linked to depressive symptoms, as extensively described in Chapter 1 (Mosiołek et al., 2021; Maes, 2011; Maes et al., 2011; Gałecki et al., 2018; Gałecki and Talarowska, 2018a,b; Kanchanatawan et al., 2017).

Given the evidence suggesting IFN-γ's potential involvement in the pathogenesis of major depressive disorder (MDD), this cytokine has been widely studied. However, the results have been inconsistent.

While baseline IFN-γ levels have been found to be significantly higher in patients with MDD compared to healthy controls (Chen et al., 2018; Dahl et al., 2014), the results of studies of antidepressant action on these levels have been inconsistent. Studies of diluted whole blood of fluoxetine-treated patients with treatment-resistant depression showed that fluoxetine significantly decreased the production of IFN-γ (Kubera et al., 2001). Blood samples from healthy female subjects treated with TCAs - amitriptyline, nortriptyline, imipramine, and desipramine - also presented reduced IFN-γ levels when stimulated with the toxic shock syndrome toxin (TSST)-1 (Himmerich et al., 2010). While in that study no significant difference in IFN-γ levels were observed between controls and patients treated with venlafaxine, it has also been reported that venlafaxine treatment resulted in decreased IFN-γ levels after 8 weeks (Chen et al., 2018). However, treatment with paroxetine led to increased IFN-γ levels after 8 weeks of treatment, suggesting differential effects of these drugs on inflammatory factors (Chen et al., 2018). Patients who completed their treatment with SSRIs, serotonin-norepinephrine reuptake inhibitors (SNRIs), mirtazapine, benzodiazepines, lamotrigine, or psychotherapy had decreased IFN-γ levels from baseline after 12 weeks (Dahl et al., 2014). In a different study of patients treated with SSRIs, IFN-γ levels fluctuated

during the treatment and significantly varied depending on the time point at which they were measured. IFN-γ levels were higher at Week 5 compared to baseline, but decreased in subsequent weeks and were comparable to the control group at Week 52 (Hernandez et al., 2013). Treatment with SSRI sertraline also resulted in a decrease in IFN-γ levels after 6 weeks, regardless of treatment response (Brunoni et al., 2014).

These studies support the results of our drug-gene interaction pathway enrichment, suggesting a relationship between depression and IFN-α, β and γ, as well as the potential of certain antidepressants to modulate these pathways. Additionally, our results show that some antidepressants interact with the antiviral mechanism by the IFN-stimulated genes pathway, further linking their action to IFN.

3.5.1.2 Activation of NF-κB by TNF receptors

The activation of the NF-κB pathway by TNF receptors 2, which bind to TNF-α, was linked with trazodone, dusolepin, sertraline, nortriptyline, reboxetine and nefazodone in our enrichment analysis. This is consistent with studies that suggest a potential relationship between the use of antidepressants and the modulation of TNF-α levels in MDD.

The potential impact of antidepressants on TNF-α levels remains a topic of ongoing research. While some studies have found correlations between TNF levels and antidepressant treatment, others have reported no significant differences. It has previously been demonstrated that psycho-social stressors activate important inflammatory pathways, such as the activation of the transcription factor NF-κB (Miller and Raison, 2016). Chronic stress exposure has also been linked to depressive-like behaviors in rats, which are believed to be mediated by NF-κB signalling (Koo et al., 2010). Most cytokines that are increased in patients with MDD depend on the NF-κB pathway, since NF-κB is considered the main transcription factor controlling cytokine synthesis (Syed et al., 2018; Martinon et al., 2002).

Elevated TNF levels are believed to play a role as a mediator in patients with depression (Mosiołek et al., 2021). A meta-analysis of 24 studies found significantly higher concentrations of TNF-α in patients diagnosed with MDD compared to healthy individuals (Dowlati et al., 2010). While most studies have reported elevated TNF

levels in patients with MDD compared to healthy individuals (Carboni et al., 2019; Eller et al., 2008; Pérez-Sánchez et al., 2018; Almond, 2013), there are cases where no differences were observed (Fornaro et al., 2013).

TNF-α level has been shown to predict treatment response to several antidepressants. Levels in patients treated with escitalopram were significantly lower at baseline (before treatment initiation) in responders as compared with non-responders, who did not differ from healthy controls (Eller et al., 2008). Carboni et al. (2019) also found that higher baseline TNF levels were correlated with better treatment responses to paroxetine but not venlafaxine. Patients treated with paroxetine showed a significant correlation between TNF elevation at baseline and improvement on the depression assessment scales after 10 weeks of treatment. However, this was not observed in patients treated with venlafaxine. On the other hand, Fornaro et al. (2013) found that patients with lower TNF levels achieved clinical improvement with duloxetine earlier than those with higher levels.

Fluoxetine significantly lowered TNF levels in patients with MDD during the fourth week of treatment. However, these levels returned to baseline after 8 weeks of treatment (Pérez-Sánchez et al., 2018). Fluoxetine treatment has also been shown to upregulate the activities of the ERK1/2-NF-κB signalling pathway in rats (Cui et al., 2016).

3.5.1.3 Interleukin-1 family signalling

Recent research has attributed a significant role in the pathogenesis of depression to the IL-1 (IL-1) family (Mosiołek et al., 2021). IL-1β levels have been found to be higher at baseline in patients with MDD when compared to healthy individuals (Chen et al., 2018; Fornaro et al., 2013; Jazayeri et al., 2010; Pérez-Sánchez et al., 2018; Piletz et al., 2009).

The effects of antidepressant treatment on IL-1β levels have been widely studied. However, the results of these studies have been inconsistent and have varied depending on the drug used and the timing of the measurements. In several studies, antidepressant treatment has been shown to reduce the levels of IL-1β in patients with MDD (Hannestad et al., 2011; Mosiołek et al., 2021). Venlafaxine, fluoxetine, paroxetine, and sertraline have been found to decrease the IL-1β levels in patients with MDD

to levels similar to those in healthy individuals (Chen et al., 2018; Hernandez et al., 2013). Clomipramine, imipramine, and citalopram have also been shown to exert varying degrees of inhibition of IL-1β (Xia et al., 1996; Sacre et al., 2010). However, a study conducted with venlafaxine has shown that the levels of IL-1β increased in patients with MDD after 8 weeks of treatment (Piletz et al., 2009), while another study found that fluoxetine did not affect the levels of IL-1β before and after treatment (Jazayeri et al., 2010). In a separate study, patients with MDD treated with fluoxetine had higher levels of IL-1β during the fourth week of treatment compared to healthy individuals, but these levels returned to baseline after 8 weeks of treatment (Pérez-Sánchez et al., 2018).

In our pathway enrichment analysis, the above-mentioned antidepressants (venlafaxine, fluoxetine, paroxetine, clomipramine, and citalopram) did not affect IL-1 cytokine family signalling. However, trazodone and nefazodone (SARIs), dosulepin (TCA), and sertraline (SSRI), all exerted some effect in this pathway. It is clear that the levels of IL-1β are elevated in patients with MDD and are affected by antidepressant treatments in different ways.

3.5.1.4 Interleukin-3 and 5

Our results also show interaction between trazodone, dosulepin and paroxetine and the IL-3, 5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) pathways.

β common (βc) cytokines GM-CSF, IL-3, and IL-5 play a crucial role in regulating various inflammatory responses in the body. These cytokines are involved in immune regulation and differentiation, by governing the onset of inflammatory responses, but also by helping turn off chronic inflammation (Dougan et al., 2019). IL-3 is a multipotent hematopoietic growth factor, expressed mainly by activated T-lymphocytes and macrophages, while IL-5 is involved in the activation and differentiation of eosinophils (Harsanyi et al., 2022).

Little is known about IL-5 potential involvement in depressive disorder. While elevated IL-5 levels have been described in patients with MDD (Elomaa et al., 2012), a later meta-analysis found that IL-5 was not significantly altered in individuals with MDD compared to healthy controls (Köhler et al., 2017a). Compared to healthy volunteers, adolescents with major depression showed significant increases in IL-5 at baseline. Af-

ter 8 weeks of fluoxetine treatment, IL-5 levels were further increased (Pérez-Sánchez et al., 2018).

Studies have also shown that elevated levels of IL-3 are associated with depression. A meta-analysis of clinical studies found that IL-3 levels were significantly higher in patients with depression (Osimo et al., 2020). Additionally, IL-3 levels were positively correlated with depressive symptoms in schizophrenic patients (Xiu et al., 2015).

While more research is needed to fully understand the relationship between these cytokines and depression, our findings, coupled with existing literature, suggest that IL-3 and 5 could be a valuable marker of depression.

3.5.1.5 Interleukin-4 and 13

In our analysis, the antidepressants trazodone, dosulepin, sertraline, and nortriptyline interacted with genes related to IL-4 and IL-13 signalling. These cytokines play a crucial role in regulating the immune response and are associated with allergic inflammation. They are important in regulating the behaviour of lymphocytes, myeloid cells, and non-hematopoietic cells (Junttila, 2018).

In mice exposed to chronic mild stress, IL-4 overexpression has been observed in the hippocampus (Zhang et al., 2021; Wachholz et al., 2017), while rodents treated with IFN-α showed more depressive behaviours when their IL-4 microglial reactivity was reduced (Wachholz et al., 2017; Chou et al., 2016).

IL-4-/- knockout mice tested in the forced swim test and tail suspension test showed significant depression-like behaviours compared to wild-type rodents, which was not further increased by the application of IFN-α (Wachholz et al., 2017).

In clinical studies of patients with MDD, IL-4 and IL-13 levels have been found to be elevated (Pérez-Sánchez et al., 2018; Pavón et al., 2006). The relationship between peripheral IL-13 elevation and MDD has also been supported by a meta-analysis of clinical studies (Köhler et al., 2017b). Elevated IL-13 levels have also been observed in suicidal patients (Vai et al., 2021), with suicide victims presenting increased expression of this cytokine in the orbitofrontal cortex (Tonelli et al., 2008). However, some studies have reported conflicting results, with decreased levels of IL-13 being observed in MDD patients (Wong et al., 2008). Such discrepancies may be due to differences in gender,

age, or severity of the disorder.

It has also been reported that the level of IL-13 can predict differential treatment outcomes in MDD (Czysz et al., 2021). Fluoxetine treatment in adolescents with MDD was found to increase anti-inflammatory cytokines IL-4 and IL-13 (Pérez-Sánchez et al., 2018). However, SSRI treatment was observed to reduce serum levels of IL-13 in depressed subjects by 69% at week 52 (Hernández et al., 2008).

It's important to note that many studies do not specify whether individuals who died by suicide were undergoing treatment with antidepressants. It's plausible that the use of antidepressants could impact suicidality, potentially serving as a confounding factor rather than inflammatory markers.

A systematic review and meta-analysis of observational studies found that the collective use of new-generation antidepressants (including SSRIs and other newer agents like venlafaxine, bupropion, or mirtazapine) is linked to a heightened risk of suicide in adult patients, particularly after adjusting for publication bias and financial conflicts of interest (Hengartner et al., 2021). However, the association between SSRIs alone and suicide risk in depression is not definitively established in adults (Hengartner et al., 2021; Nischal et al., 2012). Nevertheless, among children and adolescents, there appears to be a slightly elevated risk of suicidal thoughts and attempts associated with antidepressant use, though not necessarily completed suicides (Nischal et al., 2012).

3.5.1.6 Interleukin-6

In our analysis, the antidepressant drug trazodone was found to be the only medication that interacts with genes related to IL-6 signalling. However, IL-6 has been linked to depression in numerous studies (Mosiołek et al., 2021).

Elevated levels of the pro-inflammatory cytokine IL-6 have been found in patients with depression compared to healthy individuals (Maes et al., 1995; Bob et al., 2010; Dowlati et al., 2010; Roohi et al., 2021; Askari et al., 2019; Pérez-Sánchez et al., 2018; Manoharan et al., 2016), highlighting the potential role of inflammation in the development and treatment of depression. However, the results of the studies assessing the effects of antidepressant drugs on IL-6 levels are inconsistent and show considerable discrepancies.

Studies have shown that SSRIs, including paroxetine and sertraline, can reduce IL-6 levels in patients with MDD (Askari et al., 2019; Dong et al., 2021; Hannestad et al., 2011). A meta-analysis of clinical trials also revealed a significant decrease in IL-6 after treatment with antidepressants (Hiles et al., 2012). However, a lack of correlation between IL-6 levels and response to treatment with venlafaxine has also been reported, along with an increase in IL-6 levels with depression assessment scale score reduction after 10 weeks of treatment with paroxetine (Carboni et al., 2019). After being treated with fluoxetine for 4 weeks, IL-6 levels in MDD patients dropped significantly compared to their levels at the start of the study. However, after 8 weeks of treatment, the IL-6 levels returned to baseline (Pérez-Sánchez et al., 2018).

While in one study the baseline IL-6 levels in patients with MDD were found to be lower in responders to treatment compared to non-responders (Dong et al., 2021), in another, pre-treatment IL-6 levels of MDD patients who responded to fluoxetine were found to be the same as those who did not respond, with neither group showing a significant decrease in IL-6 levels after treatment (Manoharan et al., 2016). In a different study, higher IL-6 level was observed in patients who responded to treatment with paroxetine and sertraline compared to those who did not respond. The baseline levels of IL-6 before treatment also showed a positive correlation with the results of the depression assessment scale score (Yoshimura et al., 2013).

3.5.1.7 Interleukin-17 signalling

IL-17A is a pro-inflammatory cytokine that has been linked to various health conditions, including autoimmune diseases, obesity-associated chronic low-grade inflammation, tumorigenesis, neurodegeneration, depression, and autism (McGeachy et al., 2019; Harsanyi et al., 2022).

Studies have shown that MDD patients that don't respond to antidepressant therapy have significantly elevated IL-17A levels, which suggests that this cytokine may be a clinically relevant marker of therapy resistance (Nothdurfter et al., 2021). This is further supported by research that has investigated the relationship between the gut microbiome and depression. It has been reported that, in mice, certain types of gut microorganisms are associated with the development of depression-like symptoms and

that the primary cells involved in these processes were IL-17A-producing T helper 17 cell (Th17) cells (Medina-Rodriguez et al., 2020).

In our analysis, only two drugs, trazodone and moclebamide, interacted with genes related to IL-17 family signalling. It is possible these drugs may have the potential to target therapy resistance by modulating the IL-17A pathway.

3.5.1.8 Interleukin-10 signalling

In our analysis, only nortriptyline was found to interact with genes related to the IL-10 family signalling pathway. IL-10 is a cytokine that is primarily responsible for suppressing inflammation, playing a crucial role in its regulation. In patients with depression, conflicting data have been reported regarding IL-10 levels. While some studies have reported no change in IL-10 levels after antidepressant treatment (Pérez-Sánchez et al., 2018; Chen et al., 2018; Halaris et al., 2015), others have reported significant effects.

In one study, the antidepressant duloxetine was found to initially decrease IL-10 levels after 6 weeks of treatment, with levels returning to baseline after 12 weeks of treatment (Fornaro et al., 2013). Another study found that in diluted whole blood of fluoxetine-treated patients with treatment-resistant depression imipramine, venlafaxine, and fluoxetine significantly increased the production of IL-10 (Kubera et al., 2001).

3.5.1.9 Interleukin-20 signalling

The findings of our research have revealed that dosulepin, reboxetine, nefazodone, and milnacipran have interactions with genes related to the IL-20 signalling pathway.

IL-20 belongs to the IL-10 family of anti-inflammatory cytokines that help regulate the immune response. IL-20 subfamily cytokines generally function to enhance innate defence mechanisms, wound healing, and tissue repair at epithelial surfaces. However, there is currently almost no data available relating IL-20 specifically to depression.

One study found no difference in IL-20 levels between suicidal and non-suicidal patients with MDD (Messaoud et al., 2022). This suggests that IL-20 levels may not play a significant role in the severity of depression. However, a case-control association study established an increased risk for MDD in relation to the IL-20 and IL-24 haplotypes, which could suggest that cytokines, including IL-20, may contribute to the

pathogenesis of MDD (Traks et al., 2008).

While the current evidence regarding the relationship between IL-20 and depression is limited, it does suggest that there may be a connection between the two.

3.5.2 Innate immune response

Inflammation has long been linked to depression, and while research in this area has mainly focused on cytokines, the broader innate immune response may also be involved (Mössner et al., 2007). The innate immune system is composed of antimicrobial peptides and myeloid lineage cells, such as macrophages, dendritic cells, and neutrophils, and acts as the first line of defence against pathogens or injury. Innate immune cells constantly survey the environment for non-self signals and use pattern-recognition receptors, such as TLRs, to recognize common microbial features or other damage signals.

3.5.2.1 TLR signalling

TLR signalling has been proposed as a potential diagnostic biomarker for identifying subtypes of inflammatory depression. Pre-clinical and clinical studies have demonstrated that TLR expression and TLR signalling regulators are associated with MDD. Additionally, TLR expression and signalling are regulated in part by miRNAs and some TLR-responsive miRNAs indirectly modulate pathways that are implicated in MDD pathophysiology (Figueroa-Hall et al., 2020).

Several clinical studies have investigated the association between TLRs and depression by analyzing TLR expression, TLR-related proteins, and cytokine induction in peripheral blood mononuclear cells, monocytes, and postmortem tissue from MDD subjects (Hung et al., 2014; Hou et al., 2018; Hung et al., 2016, 2017; Wu et al., 2015; Kéri et al., 2014). These studies have shown that TLR messenger RNA (mRNA) levels were differentially expressed in MDD compared to healthy controls and TLR4 was found to be an independent risk factor relating to the severity of MDD (Hung et al., 2014; Wu et al., 2015). Other studies have linked the TLR4 receptor has been linked to depressive symptoms and the success of treatment for such symptoms (Quave et al., 2021). TLR mRNA levels, which were increased in MDD subjects, decreased

after 4 weeks of treatment with SSRIs or SNRIs, potentially indicating a TLR-mediated anti-inflammatory role for antidepressants (Hou et al., 2018; Hung et al., 2016, 2017). Another study found that relative to healthy controls, TLR4 mRNA and protein expression were elevated in MDD patients before cognitive behavioral therapy (CBT), but decreased after treatment and were associated with more pronounced clinical improvement (Kéri et al., 2014).

Two postmortem studies investigated TLR gene and protein expression in the dorsolateral prefrontal cortex of depressed suicide, non-depressed suicide, and depressed non-suicide groups (Pandey et al., 2014, 2019). These studies revealed increased levels of TLR3 and TLR4 mRNA expression in the two depressed groups, along with increased protein concentrations of TLR3 and TLR4 in the depressed group who had committed suicide, compared to controls (Pandey et al., 2014). In a follow-up study, the expression of TLR2, 3, 4, 6, and 10 protein was increased in only the group of depressed individuals who had committed suicide compared to the control group. Additionally, the expression of TLR2 and TLR3 mRNA was increased in the depressed suicide group, and the expression of TLR3, 4, and 7 mRNA was increased in the depressed non-suicide group compared to the control group (Pandey et al., 2019). Another postmortem study measured TLR4 protein concentration and TLR pathway mediators in antidepressant-treated and antidepressant-free MDD. The study found that there were no significant changes in TLR4 protein expression in the dorsolateral prefrontal cortex, but there were increases in TLR-mediated signalling proteins, with no effect of antidepressant treatment observed (Martín-Hernández et al., 2018).

Our analysis has shown that trazodone, reboxetine, and moclobemide interact with TLR 2 and 3 cascades, but not with TLR 4, 5, 7/8, and 10 cascades. These findings, along with the available literature, suggest that antidepressants may have differential effects on the TLR cascade, potentially contributing to their antidepressant efficacy.

3.5.2.2 Neutrophil degranulation

Neutrophils are the most abundant leukocytes and play an important role in fighting infections. In response to infection, neutrophils leave the circulation and migrate towards the inflammatory focus. Activated neutrophils utilize various cytotoxic mechanisms to

restrict pathogen replication, including engulfing invading pathogens by phagocytosis, producing reactive oxygen species (ROS), and degrading them by way of releasing granule-derived antimicrobial peptides (degranulation).

In our analysis, 6 antidepressants across several classes interacted with genes linked to neutrophil degranulation. Increased neutrophil counts and percentages have been observed in the peripheral blood of patients with MDD (Darko et al., 1988; Maes et al., 1994; Irwin et al., 1987; Kronfol and House, 1989; McAdams and Leonard, 1993).

The neutrophil to lymphocyte ratio (NLR), initially developed as a simple method to assess the level of systemic inflammation in critically ill patients, has more recently been employed to assess systemic inflammation in psychiatric patients (Brinn and Stone, 2020). Higher depressive scores are associated with higher NLR levels in depressive patients. In fact, NLR of more than 1.57 was found to be an independent predictor of severe or very severe depression (Aydin Sunbul et al., 2016). Significant differences in NLR were also found between non-depressed and depressed groups in males, but not in females (Kinoshita et al., 2022). Several meta-analyses have found that subjects with MDD have higher NLRs than healthy controls (Mazza et al., 2018; Zorrilla et al., 2001). Non-medicated patients with MDD have also been shown to express higher NLRs than healthy individuals, while medicated MDD patients taking SSRIs have normalized NLRs (Demircan et al., 2016). In patients with MDD, NLR has also been found to be a significant predictor of suicide risk (Ekinci and Ekinci, 2017). Neutrophil levels were also found to be higher in patients with first-episode and recurrent MDD compared to controls at baseline. After 6 weeks of treatment, improvement in depression assessment scale scores correlated with changes in neutrophil counts in first-episode MDD, suggesting that innate immunity may be involved in the early stages of MDD (Singh et al., 2022). However, there is also data pointing to a lack of a relationship between the severity of depression and NLR in patients with MDD (Kayhan et al., 2017).

Besides studies of NLR, a genetic association study of 382,485 participants also found an association between depression polygenic scores and white blood cell count, with neutrophils being the main driver of the association (Sealock et al., 2021). The

role of neutrophil phagocytosis and reactive oxygen species (ROS) production in depression has also been investigated. One study found that phagocytosis was impaired in polymorphonuclear cells, including neutrophils, in depressed individuals, but this impairment was normalized in response to effective therapy, but not in non-responders. Another study of depressed patients found that neutrophil phagocytosis was reduced during the acute phase of the disease, but returned to normal levels following remission (McAdams and Leonard, 1993). While an earlier study demonstrated that chemotaxis, superoxide release, and phagocytic capacity of circulating neutrophils were similar across diverse subtypes of depression and healthy volunteers (Maes et al., 1992a), a later meta-analysis has reported decreased cellular neutrophil function in MDD patients (Zorrilla et al., 2001).

Neutrophil functions such as phagocytosis and ROS production have also been investigated in MDD patients. In depressed individuals, phagocytosis was impaired in polymorphonuclear cells, including neutrophils, and was normalized in response to effective therapy, but not in non-responders (O'Neill and Leonard, 1990).

These studies consistently show elevated levels of neutrophils and altered ratios between neutrophils and lymphocytes in patients with MDD. The normalization of NRL observed in patients taking SSRIs further supports the importance of considering systemic inflammation in the treatment of MDD. Given that in our analysis several classes of antidepressants interacted with the neutrophil degranulation pathway, it is possible that different classes of antidepressants may have varying effects on neutrophil action in MDD.

3.5.2.3 FC epsilon receptor (FCER1)

Our research also revealed a link between 5 antidepressants and Fc epsilon receptor signalling. The high-affinity immunoglobulin E (IgE) receptor, also known as FcεRI, or Fc epsilon RI, is the high-affinity receptor for the Fc region of IgE, an antibody isotype involved in the allergy disorder. Activation of mast cells through FCERI-bound antigen-specific IgE causes the release of potent inflammatory mediators, such as histamine and cytokines. Mast cells are distributed in tissues throughout the human body and have long been recognized as playing important roles in inflammatory and immediate

allergic reactions.

There have been numerous reports documenting a rise in the occurrence of anxiety and/or depression in individuals with asthma, rhinitis, and eczema (Hashizume et al., 2005; Goodwin et al., 2004; Katon et al., 2004; Timonen et al., 2001, 2003, 2002; Buske-Kirschbaum et al., 2001; Wamboldt et al., 2000; Michel, 1994; Klokk et al., 2007). It's suggested that IgE-mediated mechanisms underlie the associations between symptoms of mental disorders and these conditions (Hashizume et al., 2005; Timonen et al., 2001, 2002; Gupta and Gupta, 2003; Arima et al., 2005; Bell et al., 1990). However, evidence for this association has been scarce.

One study compared the levels of different immunoglobulins (including total IgE levels) in psychiatric inpatients and healthy controls and found no significant differences between the groups (Sugerman et al., 1982). Another investigated correlations between total IgE and state and trait anxiety in patients with atopic dermatitis. A positive correlation was found between IgE and trait anxiety, which is characterized by a stable personality attribute affecting how events are experienced, whereas a negative correlation was found between IgE and state anxiety, defined as a more transient reaction to an adverse situation (Hashizume et al., 2005). A negative correlation was also found between total IgE and depression in patients with atopic dermatitis (Arima et al., 2005).

In study of 374 women screened for total and allergen-specific IgE, no association between case level or symptom load of anxiety/depression and total or allergen-specific IgE was found (Klokk et al., 2007). In patients with a history of prior suicide attempts and decompensation of mood disorders, there were no statistical differences in IgE levels between allergen-sensitive and non-sensitive patients (Postolache et al., 2008). Another study explored the connection between the presence of allergen-specific IgE and changes in allergy symptoms with worsening depression scores in depressed-sensitized individuals. The results showed worsening in total depressive scores from low to high pollen exposure was greater in allergen-specific IgE-positive patients as compared to allergen-specific IgE antibody-negative patients, linking specific IgE antibodies to exacerbation of depression (Manalai et al., 2012).

Overall, while it has been reported that individuals with asthma, rhinitis, and eczema have higher levels of anxiety and depression compared to healthy individuals, and the majority of the proposed causes involve IgE, there is limited support from the existing literature. However, it's worth noting that our results point to the involvement of the high-affinity IgE receptor FcεRI in the mechanism of action of several antidepressants, further establishing the need for empirical examination of the hypothesized association between IgE and depression in the general population.

3.5.2.4 Complement cascade

The complement cascade is a crucial component of the innate immune system that enhances the ability of antibodies and phagocytic cells to remove harmful microbes and damaged cells from the body. The cascade involves the synthesis of small proteins by the liver and their subsequent circulation in the blood in an inactive form. Upon stimulation by various triggers, proteases within the system cleave specific proteins to release cytokines, starting a chain reaction. The end result is the activation of phagocytes to clear foreign and damaged material, inflammation to attract additional phagocytes, and the activation of the cell-killing membrane attack complex (Mold et al., 1999).

In our study, we observed that trazodone, dosulepin, and reboxetine interact with genes involved in regulating the complement cascade. While the exact nature of this interaction and its implications are not clear in our analysis, CRP, an acute-phase serum protein involved in the activation of the complement cascade has been previously linked to depression. CRP is a mediator of innate immunity used in psychiatry as a marker of chronic inflammation (Osimo et al., 2020). Cross-sectional studies have confirmed that the levels of CRP are higher in patients with depression compared to controls (Howren et al., 2009; Haapakoski et al., 2015).

However, studies of the effect of antidepressants on CRP levels have shown conflicting results. In some studies, baseline CRP levels have been found to correlate significantly with treatment response at 2 weeks, with higher CRP values being associated with lower treatment efficacy (Chang et al., 2012). After 6 weeks of treatment with fluoxetine or venlafaxine, CRP levels have been shown to increase significantly (Chang et al., 2012). In the case of paroxetine treatment, elevated CRP levels were correlated

with a reduction in depressive symptoms (Carboni et al., 2019; Mocking et al., 2017). However, in a group of MDD patients treated with SSRIs for 6 weeks, CRP levels at the end of the study were significantly lower than those observed on admission and decreased to values comparable to those in the control group (Tuglu et al., 2003). A meta-analysis of nine studies assessing the effects of antidepressant drugs (SSRIs, SNRIs, and TCAs) on CRP levels did not show any statistically significant effect of the treatments on CRP (Więdłocha et al., 2018).

3.5.3 Adaptive immune response

Recent studies have suggested that acquired immune responses may also be involved in the parthenogenesis of MDD (Mössner et al., 2007; Haapakoski et al., 2015). Increased markers of T cell activation, such as soluble IL-2 receptor in depressed patients (Maes et al., 1995; Sluzewska et al., 1996), provide further evidence for the involvement of acquired immune responses.

Cytokines, which play a significant role in MDD, are generated by both innate and adaptive immune responses. While the innate immune response occurs quickly, the adaptive immune response, composed of lymphocytes and secreted antibodies, and relying on the proliferation and differentiation of antigen-specific T cells and B cells, takes time to be mounted. This system is responsible for immunological memory, providing a pathogen-tailored immune response, which is critical in the case of re-exposure to the same antigen (den Haan et al., 2014).

Our analysis of drug-gene interactions revealed that antidepressants interact with a number of adaptive immune system pathways. Of the 20 antidepressants studied, 12 interacted with pathways related to antigen presentation, 10 with pathways related to T-cell receptor (TCR) signalling, and 8 with B-cell receptor (BCR) signalling. T cells and B cells are activated upon recognition of antigens by their respective TCR or BCR. Furthermore, some antidepressants were found to interact with pathways related to immunoregulatory interactions between Lymphoid and non-Lymphoid cells. These pathways refer to receptors and cell adhesion molecules that play a role in modifying the response of lymphoid origin cells, such as B, T, and NK cells, to self, tumor, and pathogen antigens.

A number of studies have shown evidence of activation of cell-mediated adaptive immunity in patients with depression (Medina-Rodriguez et al., 2018). Elevated CD4+/CD8+ T cell ratios (a higher percentage of CD4+ T cells and a lower percentage of CD8+ T suppressor cells) and increased levels of activated T cells have been found in MDD patients compared to healthy controls (Darko et al., 1988; Maes et al., 1992b; Tondo et al., 1988; Maes et al., 1992a; Robertson et al., 2005). However, not all the findings are consistent. A meta-analysis by Zorrilla et al. (2001) indicated a reduction in T cell proliferative response to mitogen. Other studies have also found a decrease in the number of circulating T cells and a reduction in regulatory B cells, suggesting a dysregulation of the adaptive immune system (Ahmetspahic et al., 2018). Furthermore, MDD patients have been found to have T cells with reduced responsiveness to stimulation, indicating an immunosuppressed phenotype of T cells in MDD (Herbert and Cohen, 1993; Zorrilla et al., 2001; Irwin and Miller, 2007). Studies have shown that both Th (CD4+) and T cytotoxic (CD8+) cells have altered activities in MDD patients. The increase of the ratio of CD4+ T cells relative to CD8+ T cells, decrease in circulating regulatory T cells (Li et al., 2010; Martino et al., 2012; Toben and Baune, 2015), an increase in the Type 1 helper T cells (Th1)/Type 2 helper T cells (Th2) ratio (Myint et al., 2005), increased circulating Th17 cells (Chen et al., 2011). The results suggest a significant transformation of the Th compartment in individuals with MDD, potentially driven by the cytokine environment present in those with MDD (Medina-Rodriguez et al., 2018).

Depressive-like behaviors in mice have been associated with elevated levels of Th1 and Th17 cells in the brain (Beurel et al., 2013). This same study found that administering Th17 cells alone was enough to induce depressive-like behaviors in mice. Moreover, T cells appear to help regulate mood in mice by contributing to an adaptive response to stress (Herkenham and Kigar, 2017).

T cells have been demonstrated to be important for optimal brain function through their neuroprotective properties (Miller, 2010; Filiano et al., 2017), ongoing immune surveillance of the brain (Lewitus and Schwartz, 2009), and promotion of neurogenesis, cognitive function, and mood (Ziv and Schwartz, 2008; Schwartz and Kipnis, 2011;

Herkenham and Kigar, 2017). This evidence supports the concept of ”protective autoimmunity,” where T cells play a crucial role in maintaining brain health and function (Rook and Lowry, 2008; Lewitus and Schwartz, 2009; Lewitus et al., 2009).

The relationship between antidepressant classes and the adaptive immune system is complicated and poorly understood, with few studies conducted in this area. Treatment with SSRIs in patients with MDD has been shown to restore the Th1/Th2 imbalance observed in MDD, potentially through increasing regulatory T cells levels (Himmerich et al., 2010). A reduction in the number of circulating NK cells in MDD patients after 4 to 12 weeks of SSRI treatment has been reported, with no change in other lymphocyte subsets (Frank et al., 2004; Gladkevich et al., 2004; Ravindran et al., 1995; Rothermundt et al., 2001). MDD patients showed an increase in NK cells and B cells after 20 weeks of SSRI treatment (Hernandez et al., 2010).

Activated mature B lymphocytes proliferate in a time-dose-dependent manner to either 5-HT or 5-HT1A receptor agonist concentrations (Iken et al., 1995). As a result, it has been suggested that the increase in extracellular 5-HT levels caused by long-term SSRI treatment may trigger the proliferation of B lymphocytes in patients with MDD. The number of NK cells might also increase due to stimulation of their serotonergic receptors caused by the increase in 5-HT levels resulting from long-term SSRI treatment (Hernandez et al., 2010).

A study of nortriptyline monotherapy in MDD patients revealed changes in lymphocyte activation and decreased NK cell numbers and function during depression, with significant decreases in T cells and T-cell mitogen responses after 6 weeks of treatment and concurrent clinical improvement (Schleifer et al., 1999). Venlafaxine treatment in elderly patients with depression was associated with changes in lymphocyte gene expression, with genes involved in cell survival, neural plasticity, and signal transduction being differently expressed (Kálmán et al., 2005).

3.5.4 Conclusion

The interaction between antidepressants and the immune system is complex, as evidenced by our results of drug-gene interaction pathway enrichment and the supporting studies cited above. Our results point to antidepressant interaction with a large number

of pathways that varies between classes and even within the same class. Considering the literature discussed above, these results are to be expected. It is evident that different classes of antidepressants may interact with different components of the immune system. Not only that but, within the same class, immunomodulatory action can vary. Different components of the immune system may have varying reactions to different antidepressants, suggesting differential effects of these drugs on inflammatory responses. While SSRIs and TCAs are the most commonly prescribed and studied antidepressants, SARIs, in particular Trazodone, emerged as a candidate with high immunomodulatory potential. Contrastingly, SSRIs and TCAs had mixed results, with some drugs exhibiting high immunomodulatory potential and others none. While the effects of antidepressant treatment on the immune system have been widely investigated, the results of these studies are also at times inconsistent and have varied depending on the drug used and the timing of the measurements.

The cytokines often talked about in the psychoimmunological literature can generally be classified according to their immunological function into four categories (Himmerich et al., 2019):

- Th1 cytokines (IL-2, IL-12, IFN-γ) which fuel the Th1 branch of the immune system and lead to cytotoxic cell contacts.
- Th2 cytokines (IL-4, IL-5, IL-13) which stimulate the Th2 branch of the immune system and induce the production of antibodies.
- Pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-17, IL-21, IL-22, IFN-α, TNF-α) which promote inflammation.
- Anti-inflammatory cytokines (IL-10, TGF-β) which are influenced by regulatory T cells and prevent inflammatory processes from escalating.

Interestingly, the antidepressants in our study were shown to interact with genes related to all four classes of cytokines, showing great diversity in targets. Although some studies have presented conflicting evidence, overall, levels of both pro- and anti-inflammatory cytokines have been found to be elevated in MDD, and antidepressants

generally lead to a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines.

Research has flagged certain markers, such as TNF alpha and IL-6, as predictors of antidepressant effectiveness, while others, like IL-17A, have been suggested as markers of therapy resistance. All these pathways were predicted to be targets of antidepressant action by our enrichment analysis. Interestingly, in our study, several antidepressants were found to interact with genes related to the IL-20 signalling pathway, an area of cytokine research that is still relatively unknown. Similarly, the relationship between antidepressants and the adaptive immune system is poorly understood, and these pathways may warrant further investigation.

While some components of the innate immune response highlighted in our enrichment analysis, such as TLR and neutrophil action, have been clearly linked to MDD, others, such as FCER1/IgE and the complement cascade/ CRP, require additional research to fully understand their role in this disorder. Of particular interest to this thesis is the interaction between MDD and neutrophils, as we have shown elevated levels of neutrophils in the gut of zebrafish displaying behavioural alterations following exposure to the pro-inflammatory chemical dextran sodium sulfate (DSS) (Chapter 2.

The enrichments results obtained for dexamethasone further support the validity of the method utilized in this chapter. Dexamethasone interacted with the various immune pathways linked to its mechanisms of action, which were also predicted to be targeted by several antidepressants. The only immune pathway affected by dexamethasone but not by the antidepressants examined was IL-12. IL-12 has been linked to depression and has been found to be elevated in depressed individuals, with levels decreasing after antidepressant treatment (Kim et al., 2002; Lee and Kim, 2006; Sutcigil et al., 2007; Nowak et al., 2019; Schmidt et al., 2014; Hou et al., 2019). This correlation has been confirmed by a meta-analysis of 82 studies conducted in 2017 (Köhler et al., 2017b). However, a 2020 systematic review and meta-analysis investigated whether measuring baseline peripheral cytokine levels, including IL-12, could predict antidepressant treatment response, and found no significant difference in cytokine levels between individuals who responded to treatment and those who did not

(Liu et al., 2020).

Perturbation databases such as LINCS offer a wealth of opportunities for computational drug discovery approaches by enabling pharmacogenomics that extends beyond classical pharmacology (Musa et al., 2018). Several studies have utilized the L1000 CMap 2.0 data set to identify small molecules for drug discovery and repurposing (Keenan et al., 2019). One of these studies identified CGP-60474 as a highly potent anti-sepsis agent, based on a signature matching strategy (Han et al., 2018). L1000 CMap 2.0 queries were also utilized to prioritize small molecules for cystic fibrosis (Wang et al., 2018), melanoma (Fagone et al., 2017), and pancreatic ductal adenocarcinoma (Er et al., 2018). A small-molecule kinase inhibitor was discovered to attenuate the spread of Ebola in human cell lines (Duan et al., 2016) and dutasteride predicted anti-neuroinflammation activity was validated in vitro and in vivo (Luo et al., 2021). Cell viability measurements of six compounds confirmed four of the six toxicity predictions from the LINCS-L1000 dataset (Alföldi, 2018). Additionally, the L1000 dataset was also included in a machine learning framework to enhance the forecasting of side effects for FDA-approved drugs (Wang et al., 2016).

However, the LINCS L1000 database comes with some known limitations, such as dosage-dependent conditions and the uncertainty of applying cell lines expression patterns to human systems. Although drug perturbation signatures have been employed to identify links, similarities, and dissimilarities between diseases, drugs, genes, and pathways, our grasp of their full potential remains incomplete. Methodologies that incorporate other omics, such as metabolomics and proteomics, as well as dynamic or longitudinal data, could help us gain a more thorough comprehension of drug mechanisms. These approaches could broaden the limited perspective provided by the single time point of transcriptomic responses (Musa et al., 2018). Several methods and algorithms have also been specifically developed to enhance the processing of L1000 data. For example, a two-dimensional spatial bias was detected in L1000 assays conducted in 384-well plates, and a method was established to correct for these biases (Lachmann et al., 2016). Additionally, a method called model-based clustering with data correction was designed to identify and correct artefacts created in the way expression

levels are assigned to genes using the L1000 assay (Young et al., 2016).

Another major limitation of LINCS-L1000 is the utilization of cancer cell lines. This aspect adds to the challenge of extrapolating findings to non-cancer contexts and may introduce biases in the interpretation of results. Despite this limitation, the use of cancer cell lines offers certain advantages, such as their widespread availability and well-established characterization. Despite their malignant phenotype, many fundamental cellular processes and signalling pathways are conserved between cancerous and normal cells. Consequently, studying the response of cancer cell lines to non-cancer-related pharmaceuticals can offer valuable insights into the potential effects of these drugs on normal cellular physiology. Nonetheless, the extent to which these findings translate to the inflammatory markers under investigation in this chapter remains uncertain.

LINCS provides measurement data for various experimental factors, including multiple cell types, doses, and time points. While traditional consensus views aggregate this data, intentionally ignoring possible cell-line-specific effects of the drugs (Parkkinen and Kaski, 2014), our method is capable of distinguishing cell-line-specific effects. It has been reported that only a minority of compounds in the LINCS database yield a similar signal across cell lines (Subramanian et al., 2017). Comparing the signatures of 2,429 drugs across 9 cancer cell lines, out of 58% of the compounds that were active in at least 3 cell lines, 26% (corresponding to 15% of all compounds) produced highly similar signatures across the entire panel, whereas the remainder produced a diversity of cellular signatures. It was also reported that connections with support across multiple cell types tended to target core cellular processes (e.g., ribosomal function and proteasome complex), whereas compounds with reproducibly cell-type-selective patterns of connectivity tended to target more specialized mechanisms. For example, connectivity between multiple-glucocorticoid receptor agonists was strongest in those cell types in which the glucocorticoid receptor was expressed. The diversity in response across cell lines for the same tested antidepressant seen in our study is consistent with these reports. We observed connectivity with immune pathways across several cell-lines, but those pathways were not normally activated across cell lines responding to each

antidepressant.

While recent methods using LINCS L1000 data have shown great promise, it's important to note that further research is needed to fully comprehend the mechanisms underlying these interactions and their effects on the efficacy of antidepressants in treating depression. Ongoing research aims to extend the connectivity mapping concept and methodology, but certain aspects require further investigation. As of yet, the field lacks systematic evaluations of the new approaches, resulting in imprecise measurement of the advantages and disadvantages of different methods (Musa et al., 2018).

It is worth noting that it is not possible to extrapolate from our results which specific immune pathways are up- or down-regulated by antidepressant treatment. However, future work looking at differential gene expression signatures for specific drug-gene interactions linked to immune pathways of interest could shed light on the effect of antidepressants on pro- and anti-inflammatory mechanisms.

By analyzing transcriptomic signature data from a diverse array of antidepressants and assessing their interactions with relevant biological pathways, we gain insights into the potential immunomodulatory effects of these compounds. This information allows us to identify specific antidepressants that exhibit distinct interactions with immune-related pathways, indicating their probable efficacy in modulating inflammatory processes associated with MDD. By ranking antidepressants with robust anti-inflammatory profiles, we can strategically design in vivo experiments aimed at elucidating their impact on immune dysfunction within the context of depression. This targeted approach not only streamlines the selection process for experimental investigations but also lays the groundwork for developing more tailored and effective therapeutic strategies for MDD.

Validating predicted drug-gene interaction enriched pathways experimentally is also crucial. In particular, studies involving primary cells from patients with MDD, treated with the antidepressants predicted to have high immunomodulatory potential, could greatly improve the understanding the aetiology of MDD. By providing a more targeted approach to research, the pathways identified in this chapter may provide im-

portant clues for identifying new therapeutic targets for MDD and help to accelerate progress in the development of more effective treatments.

The zebrafish model developed in Chapter 2 provides a platform for testing the anti-inflammatory potential of the antidepressants identified in this Chapter. By administering these antidepressants to zebrafish previously subjected to inflammation-inducing agents such as DSS, their ability to mitigate the behavioral disruptions caused by inflammation can be assessed, providing insights into their efficacy as potential antidepressants.

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Chapter 4

In vivo validation of computationally predicted anti-inflammatory properties of antidepressants

4.1 Abstract

Based on the computational analysis conducted in Chapter 3, trazodone interacted with the largest number of pathways linked to the immune system, while citalopram, ranked amongst the lowest for anti-inflammatory potential. This chapter aimed to investigate the anti-inflammatory properties of these two antidepressants, in zebrafish exposed to the pro-inflammatory chemical dextran sodium sulfate (DSS). It was hypothesised that by co-exposing zebrafish to dextran sodium sulfate (DSS) and trazodone, the latter could rescue the behavioural responses induced by DSS, while citalopram was not expected to exert this effect due to its low predicted anti-inflammatory properties.

Although the behavioural outcomes induced by DSS were in line with previous experiments, the impact of trazodone and citalopram on behaviour remains uncertain. However, both drugs demonstrated clear anti-inflammatory effects by rescuing increased neutrophil infiltration to the gut in zebrafish exposed to DSS. While this provides new evidence for the role of neutrophils and inflammation in the pathophysiology of depression, it also indicates potential limitations of the drug-gene interaction pathway enrichment analysis conducted in Chapter 3.

Additional trials to validate these results and rule out the effect of inter-individual variability may offer additional insights into the effectiveness of the prediction method used in the computational analysis. Overall, this study highlights the potential of trazodone and citalopram as anti-inflammatory agents in the treatment of depression and offers new insights into the role of inflammation in the pathophysiology of depression. Further investigation of the effects of these drugs on the wider immune response is necessary to fully understand their anti-inflammatory properties.

4.2 Introduction

Based on the computational analysis conducted in Chapter 3, two antidepressants were chosen for the *in vivo* validation studies. In the analysis conducted in the previous chapter, trazodone, a serotonin antagonist and reuptake inhibitor (SARI), interacted with the largest number of pathways linked to the immune system, while citalopram, a selective serotonin reuptake inhibitor (SSRI) ranked amongst the lowest for anti-inflammatory potential. In Chapter 2, we described how the pro-inflammatory chemical DSS simultaneously induces inflammation in target organs (*i.e.* intestine) and behavioural effects. Here, we hypothesise that by co-exposing zebrafish to DSS and trazodone, the latter can rescue the behavioural responses induced by DSS. On the other hand, citalopram was not expected to exert this effect due to its low predicted anti-inflammatory properties. Our findings from the drug-gene interaction pathway enrichment and the referenced studies in Chapter 3 indicate that the relationship between antidepressants and the immune system is complex. Antidepressants interact with a large number of pathways that vary between classes and even within the same class. Experimental validation is crucial to support these computational predictions and may provide important clues for identifying new therapeutic targets for major depressive disorder (MDD) and help to accelerate progress in the development of more effective treatments.

4.2.1 Citalopram

SSRIs are the first choice pharmacological treatment option for major depression. Citalopram hydrobromide is the most prescribed antidepressant in the UK from 2009

to 2017, having almost doubled the number of items prescribed from 2007 to 2017 (7,781,596 to 14,289,426) (Prescribing and Medicine Team, 2018). In a network meta-analysis of double-blind, randomised controlled trials with 21 antidepressants, citalopram was shown to have a lower dropout rate than other antidepressants, while having average efficacy (Cipriani et al., 2018).

It acts as an antidepressant by potentiating serotonergic activity in the central nervous system, inhibiting the reuptake of serotonin while having minimal effects on norepinephrine, and dopamine neuronal reuptake. It has a low affinity for muscarinic acetylcholine receptors and mild antagonist actions at histamine receptors, but no significant effect on α- and β-adrenergic receptors, dopamine-1, dopamine-2, γ-aminobutyric acid, opioid, or benzodiazepine receptors (Richelson, 2003; Hyttel, 1982).

As demonstrated and discussed in the previous chapter, several SSRIs have been demonstrated to have anti-inflammatory potential. Experimentally, citalopram has been shown to attenuate lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production *in vitro* at concentrations known to occur within the brain with conventional pharmacological treatment (Tynan et al., 2012). *In vitro* exposure to citalopram also inhibited mitogen stimulation of cultured human lymphocytes (Mårtensson and Nässberger, 1993). However, longer durations of exposure to low concentrations created a situation in which the drug was moderately pro-inflammatory (Tynan et al., 2012). This is consistent with results obtained by Tynan et al. (2012) with other SSRIs, as well as existing literature, showing that under certain conditions, antidepressants possess the capacity to exert both pro- and anti-inflammatory immunomodulatory properties (Abdel-Salam et al., 2003).

While protein kinase A (PKA) has been implicated in the mechanism through which citalopram exerts its immunomodulatory action, as inhibition of this kinase with ribose-modified cyclic adenosine monophosphates (Rp-cAMPs) attenuated its anti-inflammatory response (Tynan et al., 2012), this role only partially explains the effects, and other mechanisms are clearly at work. Citalopram inhibits cytokine production induced by toll-like receptors (TLRs) 3, 7, and 9 in murine bone marrow–derived macrophages, as well as cytokine production induced by TLR-3 and TLR-8 in primary

Table 4.1 – Summary of behavioural effects of citalopram in zebrafish as described in literature. No Observed Effect Concentration (NOEC); Lowest Observed Effect Concentration (LOEC)

Life stage	Exposure	NOEC (µg/L)	LOEC (µg/L)	Effects on behaviour	Reference
Adult (3-4 months)	3 h	500	1000	↓ locomotor activity	(Kulikova et al., 2021)
Adults	5 min	100000	30000	↑ geotaxis	(Karakaya et al., 2021)
Embryo to adult	180 d	1	10	↓ reproductive behaviour	(Hong et al., 2022)
Juvenile (6 weeks)	21 d	1	10	↓ locomotor activity	(Hong et al., 2021)
		10	100	↓ individual social behaviour	
			≤1	↑ collective behaviour	
Embryo	21-47 h	50000	100000	↓ coiling activity	(Zindler et al., 2019)
Embryo	to 107 hpf	41	499	↓ visual motor response	(Zindler et al., 2020)
Embryo	to 144 hpf	98	373	↓ dark swimming activity	(Bachour et al., 2020)
Embryo	to 96 hpf	≤20	20	↓ dark swimming activity	(Steele et al., 2018a)

human macrophage colony-stimulating factor (M-CSF) macrophages and rheumatoid arthritis synovial fibroblasts. Additionally, citalopram inhibits spontaneous cytokine production in human rheumatoid arthritis synovial membrane cultures (Sacre et al., 2010).

The effects of antidepressants on fish have been widely reported. Citalopram has been demonstrated to antagonize serotonin reuptake transporters and provoke a wide variety of behavioural effects in several fish species (Bachour et al., 2020), including japanese medaka larvae (Chiffre et al., 2016), crayfish (Buřič et al., 2018), three-spine stickleback (Kellner et al., 2015), guppy (Olsén et al., 2014) and zebrafish (Table 4.1), at concentrations well below the LC50 reported for each respective species.

In zebrafish, citalopram up to 8mg/L did not cause elevated mortality or malformation in larvae exposed from 3 to 144 hours post fertilization (hpf) (Bachour et al., 2020), while larvae exposed from early embrionic stages to 96 hpf have reported LC50s of 38.4 mg/L (Steele et al., 2018a) and 64.2 mg/L (Zindler et al., 2019).

The therapeutic hazard value for citalopram in zebrafish was calculated by Steele et al. (2018a) to be 0.02 mg/L. This value is based on mammalian pharmacology and physicochemical data for the drug (Brooks, 2014) and represents the predicted water concentration leading to human therapeutic doses of the pharmaceutical in fish, calculated based on blood:water chemical partitioning coefficients (Fitzsimmons et al., 2001; Huggett et al., 2003).

4.2.2 Trazodone

Trazodone is an antidepressant classed as a SARI, which works by inhibiting both serotonin transporter and serotonin type 2 receptors. Trazodone inhibits the reuptake of serotonin and blocks the histamine and α-1-adrenergic receptors, as well as inducing significant changes in serotonin presynaptic receptor (Shin and Saadabadi, 2022). However, the full spectrum of trazodone's mechanism of action is not yet fully understood.

While it has previously been reported that trazodone is comparable in efficacy to other drug classes, such as tricyclic antidepressants (TCAs), SSRIs, and serotonin-norepinephrine reuptake inhibitors (SNRIs) in treating major depressive disorders (Fagiolini et al., 2012), a network meta-analysis of double-blind, randomised controlled trials with 21 antidepressants, found trazodone to be less efficacious than other antidepressants, as well as being among the drugs with the highest dropout rates (Cipriani et al., 2018).

Besides treatment of depression, trazodone is commonly used off-label for other conditions (Settimo and Taylor, 2018), mainly to treat insomnia (Wong et al., 2017), but also to treat the behavioural and psychological symptoms of dementia in Alzheimer's disease (López-Pousa et al., 2008) and in fronto-temporal dementia (Lebert et al., 2004). It has previously been shown to be of benefit in models of Huntington's disease (Kumar et al., 2011), and to prevent neurodegeneration in mice (Halliday et al., 2017). In the carrageenan model of paw inflammation in rat, trazodone (Abdel-Salam et al., 2003; Hajhashemi and Khanjani, 2014) significantly inhibited paw oedema.

In vitro, trazodone has been shown to significantly decrease the cellular release of the pro-inflammatory cytokine interferon (IFN)-γin neuronal-like cells, as well as inhibit the production of inflammatory mediators in neuronal cells treated with LPS and TNF-α (Daniele et al., 2015a). In the same study, trazodone induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, inhibited mitogen-activated protein kinase (MAPK) p38 activation, and counteracted the activation of MAPK p38 and c-Jun N-terminal kinase (JNK) elicited by LPS and TNF-α, suggesting that the neuroprotective role of trazodone could be mediated by MAPK p38 and JNK. On healthy human

astrocytes, pre-treatment with trazodone before inflammatory insult with LPS and TNF-α, reduced the activity of inflammatory mediators, including interleukin-6, JNK and nuclear factor kappa B (NF-κB) (Daniele et al., 2015b). Trazodone also affected astrocyte metabolic support to neurons by counteracting the inflammation-mediated lactate decrease (Daniele et al., 2015b).

Experimental data in zebrafish is scarcer for trazodone than citalopram. In scn1lab s552/s552 mutant zebrafish larvae (a model of early-life seizures) exposed for 30 minutes at 5 days post-fertilization (dpf), concentrations up to 250µM (102.08 mg/L) trazodone did not alter behaviour under light and dark conditions (Grone et al., 2017). In 5 dpf scn1Lab mutant zebrafish larvae exposed for 90 minutes, trazodone led to mortality levels above 50% at 750 µM (306.2 mg/L), while 500µM (204.15 mg/L) was not toxic (Griffin et al., 2017). However, Huang et al. (2018) reported mortality in zebrafish larvae exposed from 96 to 144 hpf at much lower concentrations of trazodone at 100µM (40.83 mg/L), likely explained by the longer exposure times (48 hours versus 90 minutes). In the same setting, trazodone induced hepatotoxicity at 40µM (16.332 mg/L) and 80uM (32.664 mg/L) (Huang et al., 2018).

4.3 Methods

4.3.1 Ethics Statement

Experiments were carried out at Brunel University London under Project License and Personnel Licences granted by the UK Home Office under the United Kingdom Animals Act (Scientific Procedures).

4.3.2 Animals

All animal rearing and breeding were conducted as described in Section 2.3.

The number of biological replicates utilized in each experiment is detailed in the results section of that experiment and summarized in Appendix A. It's worth noting that the variation in the number of biological replicates is influenced by the specific goals of each experiment and the availability of zebrafish larvae.

4.3.3 Test chemicals

Citalopram hydrobromide (CAS number 59729-32-7) and Trazodone hydrochloride (CAS number 25332-39-2) were supplied by Sigma-Aldrich. DSS (CAS number 9011-18-1) was supplied by MP Biomedicals as powder.

All stocks were prepared and maintained as described in Section 2.3.

4.3.4 Exposure to pro-inflammatory chemicals and antidepressants

All exposures were conducted as described in Section 2.3. Based on the literature consulted and taking into consideration the longer exposure time of 10 days in our experiments, two range-finding experiments were conducted with concentrations of trazodone from 0.21875 mg/L to 3 mg/L, and citalopram from 0.09375 mg/L to 1.5 mg/L.

Following the range-finding experiments, two co-exposures with DSS were conducted. Each study was carried out in 2 independent experiments. In the first exposure, larvae were exposed to 330 mg/L DSS alone, and 0.75 mg/L and 1.5 mg/L citalopram alone and in combination with 330 mg/L DSS. In the second, the DSS concentrations remained the same, with citalopram replaced with 0.04 and 0.2 mg/L trazodone. For all exposure, at 72 hpf, after hatching, Tg(MPx GFP)i114 zebrafish larvae were exposed for 10 days to the selected DSS and antidepressant concentrations in recirculating system water, along with a water-only control.

4.3.5 Behaviour tracking - Locomotor test

All locomotor tests were conducted as described in Section 2.3. Briefly, at the end of the exposure time, 13 dpf zebrafish larvae underwent a 50-minute dark-light challenge. This period included a 10-minute dark acclimation period followed by a 40-minute observation period consisting of two alternating 10-minute light/dark cycles. The behavioural parameters quantified in the study included distance travelled, average speed, time spent and distance travelled in the central and outer area of the well and response to dark/light stimuli.

4.3.6 Fluorescence imaging

All *in vivo* fluorescent imaging was conducted as described in Section 2.3, using a Leica DMi8 microscope. Acquired images were analysed using Image J software to quantify the number of GFP-positive cells infiltrated in the mid-posterior region of the zebrafish intestine.

4.3.7 Statistical approach

All statistical analyses were conducted using Graphpad Prism (GraphPad Software, San Diego, California, United States). Normality was assessed using the tests available in Graphpad Prism, including Shapiro-Wilk, D'Agostino-Pearson and Kolmogorov-Smirnov tests. Normally distributed data were analysed using ordinary one-way ANOVA. Data that were not normally distributed were analysed using Kruskal-Wallis tests. Groups were compared using multiple comparison tests: Dunnett test for ranges of concentrations to correct for multiple comparisons; Fisher's Least Significant Difference (LSD) test for experiments comparing multiple compounds where each comparison stands alone. Box and whisker graphs display the median, and the error bars represent the 5-95 percentile; individual dots represent outliers. Statistical significance is reported using asterisks as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ **** $p<0.0001$.

4.4 Results

4.4.1 Behaviour and immune profile of zebrafish larvae co-exposed to Dextran Sulfate Sodium (DSS) and Citalopram for 10 days

A range-finding experiment was conducted using the concentrations of citalopram determined through a literature review. At 72 hpf, after hatching, 10 Tg(MPx GFP)i114 zebrafish larvae were loaded into each well of 6-well plates containing 5 increasing concentrations of citalopram hydrobromide (0.09375, 0.1875, 0.375, 0.75, and 1.5 mg/L) in recirculating system water, along with a water-only control. A total of 60 larvae were used, with 10 larvae per exposure group. Zebrafish larvae were exposed for 10 days and maintained as described in the methods section.

Mortality in all exposure groups ranged between 20% and 40%, with control and citalopram at the highest concentration displaying the highest mortality rates (Fig. 4.1). A low mortality rate after external feeding starts, generally up to 30%, is considered natural and acceptable. In this case, both control and 1.5 mg/L citalopram caused 40% mortality rates, while 0.75 mg/L had a lower mortality rate. As the mortality level at the highest concentration was the same as controls, mortality was attributed to natural causes due to failure to initiate external feeding. Coupled with the much higher LC50s described in the literature (25x higher), the two higher concentrations were considered safe and were selected for co-exposure with DSS.

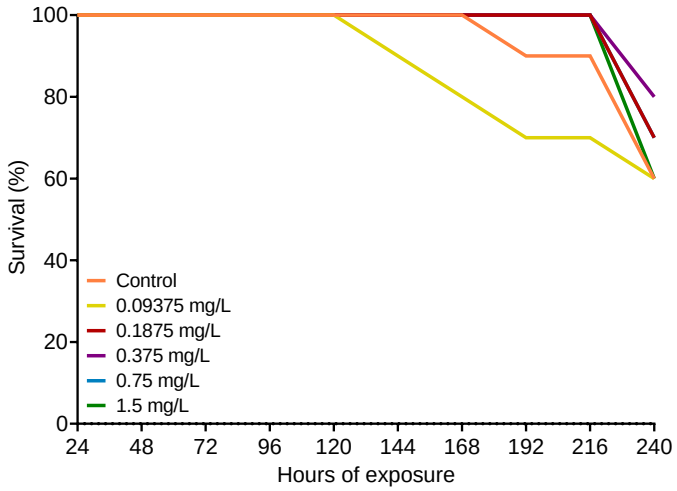


Figure 4.1 – Mortality of zebrafish after 10-day exposure to antidepressant citalopram at increasing concentrations. Larvae were introduced to the dilutions from 3 dpf. Results area for 10 larvae per group. Exposure to DSS concentrations ranging from 0.09375 to 1.5 mg/mL resulted in mortality rates under 40% for all exposure groups.

Tg(MPx GFP)i114 zebrafish were exposed for 10 days to a water control, 330 mg/L DSS alone, and 0.75 mg/L and 1.5 mg/L citalopram alone and in combination with 330 mg/L DSS. The study was carried out in 2 independent experiments with a total of 33 larvae per exposure group. At 72 hpf, after hatching, up to 9 larvae were loaded into each well of 6-well plates containing the selected concentrations in recirculating system water, along with a water-only control. A total of 198 larvae were used, with 33 larvae per exposure group. Zebrafish larvae were maintained as described in the methods section.

Exposure to DSS and citalopram alone or in combination resulted in mortality rates

comparable to controls, which displayed 30% mortality (Fig. 4.2).

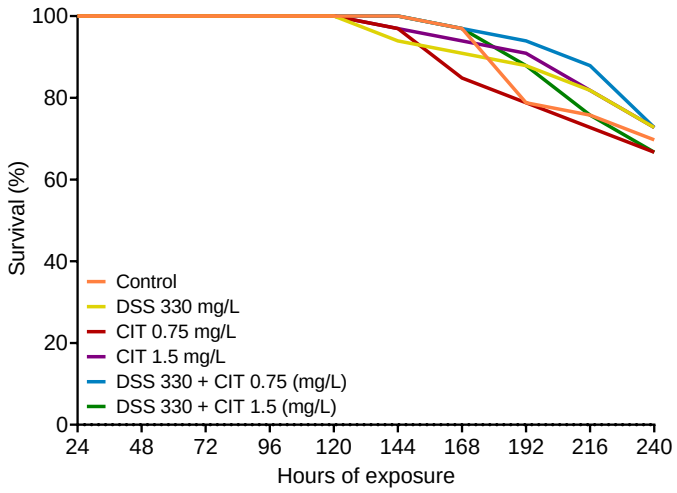


Figure 4.2 – Mortality of zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Results are for 33 larvae per group. Exposure to DSS and citalopram (CIT) alone or in combination resulted in mortality rates under 33% for all exposure groups.

DSS and citalopram alone or in combination did not affect phototaxis at the selected concentrations (Fig. 4.3, Table 4.4). Larvae co-exposed to 330 mg/L DSS with 1.5 mg/L citalopram seem to be more active in all light conditions from approximately 20 minutes from the start of the recording to the end. Breaking down these results on a plate-by-plate basis, it becomes obvious that this phenomenon was not driven exclusively by a single plate or individual experiment, as 3 plates displayed increased activity in the last 30 minutes, and 2 different plates displayed this activity even earlier starting at 20 minutes from the start. The remaining 2 plates displayed lower activity in this exposure group throughout the 50-minute period. The control group and larvae exposed to DSS alone displayed a spike in activity in the first 10 minutes in dark conditions. This was also observed in different degrees of intensity across all plates.

Compared with controls, larvae co-exposed to 330 mg/L DSS with 1.5 mg/L citalopram (Fig. 4.4) displayed an increase in distance travelled (4.908 to 9.964 meters) and average speed (1.664 to 2.563 mm/s) but these changes were not significant ($p=>0.9999$) (Tables 4.2 and 4.3). The groups exposed to 1.5 mg/L citalopram, alone or in combination with DSS, also displayed much higher variability than other groups (Tables 4.2 and 4.3). This can also be observed in average speed for larvae exposed

to 0.75 mg/L citalopram, and larvae co-exposed to 1.5 mg/L citalopram with 330 mg/L DSS. Exposure to DSS and citalopram alone or in combination did not change the distribution of movement at different speeds (Fig. 4.5).

The results for time and distance spent in the central and outer areas of the well display higher variability than other endpoints. While an increase in time spent and distance moved in the outer area of the well can be seen in larvae exposed to 0.75 mg/L citalopram, and 1.5 mg/L citalopram with or without DSS (Fig. 4.6a, Fig. 4.6b), these changes are not significant, likely due to the high variability.

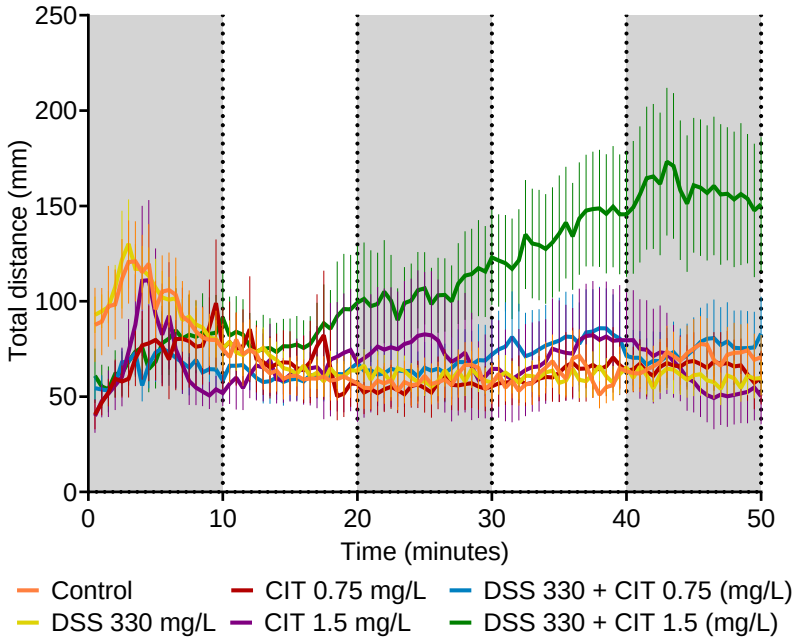
Although behaviour was not significantly altered by citalopram, the results for the imaging of neutrophil infiltration were much clearer. Consistently with previous experiments, DSS significantly increased the number of neutrophils in the gut (Fig. 4.7). This increase was not present in larvae co-exposed with citalopram, which displayed numbers comparable with controls and were significantly lower to the levels seen in DSS exposed larvae.

Table 4.2 – Descriptive statistics for total distance travelled by larval zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS.

	Control	DSS 330 mg/L	CIT 0.75 mg/L	CIT 1.5 mg/L	DSS 330 mg/L + CIT 0.75 mg/L	DSS 330 mg/L + CIT 1.5 mg/L
n	23	25	21	18	21	19
Minimum	1.976	0	1.348	0	0	1.855
Maximum	14.435	11.566	19.366	27.029	19.543	26.868
Range	12.460	11.566	18.017	27.029	19.543	25.013
Mean	4.908	4.413	5.540	6.396	5.335	9.964
Std. Deviation	3.342	2.770	5.118	7.144	5.523	8.098
Std. Error of Mean	0.697	0.554	1.117	1.684	1.205	1.858

Table 4.3 – Descriptive statistics for average speed (mm/s) travelled by larval zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS.

	Control	DSS 330 mg/L	CIT 0.75 mg/L	CIT 1.5 mg/L	DSS 330 mg/L + CIT 0.75 mg/L	DSS 330 mg/L + CIT 1.5 mg/L
n	23	25	21	18	21	19
Minimum	0.8234	0	0.5605	0	0	0.77
Maximum	3.875	4.397	5.213	4.052	5.078	5.583
Range	3.051	4.397	4.652	4.052	5.078	4.813
Mean	1.664	1.513	1.996	1.542	1.74	2.563
Std. Deviation	0.873	0.9555	1.44	1.037	1.355	1.592
Std. Error of Mean	0.182	0.1911	0.3141	0.2445	0.2957	0.3652

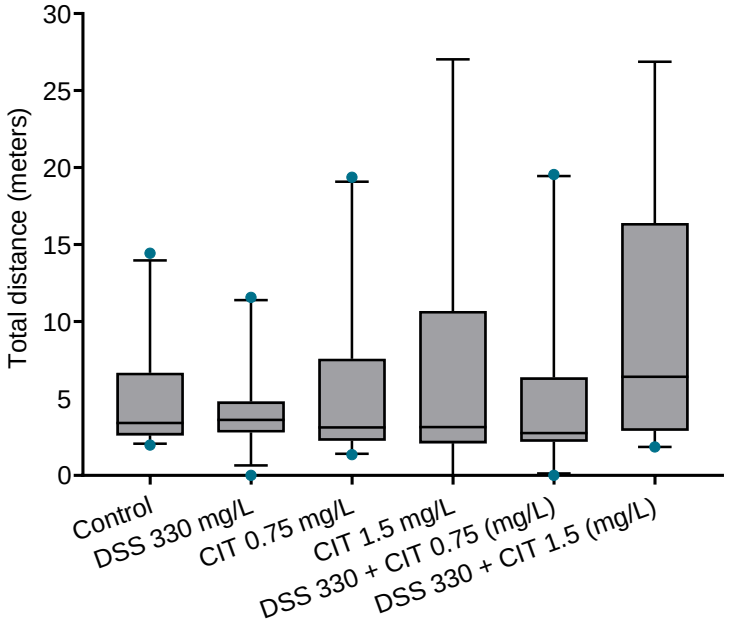


(a) Mean distance travelled by larvae under alternating dark and light conditions

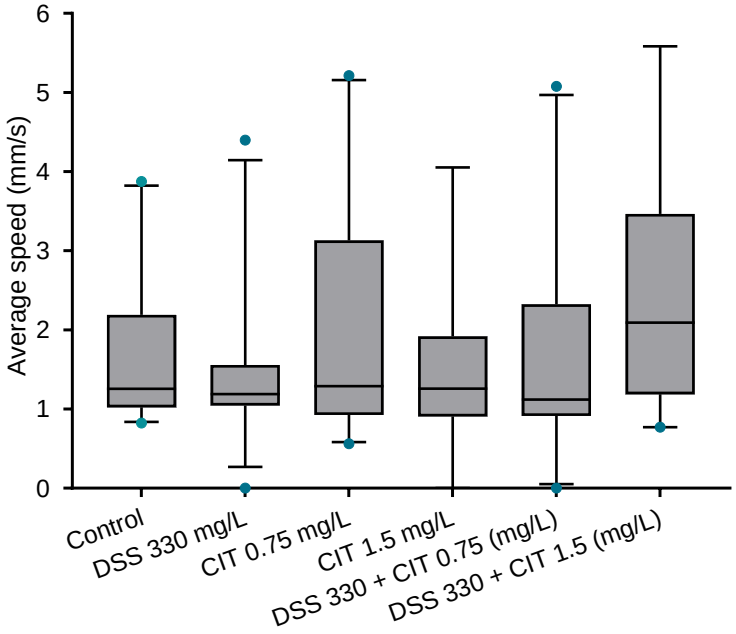
Figure 4.3 – Effect of alternating light–dark periods on locomotion in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS. Free swimming hatched larvae were introduced to the dilutions from 3 dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. The statistical methods applied are outlined in the methods section (2.3.8). Grey areas signify dark conditions. Data are presented as mean distance moved (in mm) in 30-second intervals throughout a 50-minute session. Results are for 23 (control), 25 (DSS 330 mg/L), 21 (CIT 0.75 mg/L and DSS 330 mg/L + CIT 0.75 mg/L), 18 (CIT 1.5 mg/L), and 19 (DSS 330 mg/L + CIT 1.5 mg/L) larvae per group.

Table 4.4 – Number of time points with statistically significant difference compared to control for data in Fig. 4.3. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). N=(control), 22 (0.001 mg/mL), 19 (0.0033 mg/mL), 23 (0.01 mg/mL), 24 (0.033 mg/mL), 41 (0.1 mg/mL) and 42 (0.33 mg/mL) larvae per group.

	Control *vs.* DSS 330 mg/L	Control *vs.* CIT 0.75 mg/L	Control *vs.* CIT 1.5 mg/L	Control *vs.* DSS 330 + CIT 0.75 (mg/L)	Control *vs.* DSS 330 + CIT 1.5 (mg/L)	DSS 330 mg/L *vs.* DSS 330 + CIT 0.75 (mg/L)	DSS 330 mg/L *vs.* DSS 330 + CIT 1.5 (mg/L)
Time points with statistically significant difference *vs.*control	15	0	0	6	26	3	28



(a) Total distance travelled by larvae (meters)



(b) Average speed of larvae (mm/s)

Figure 4.4 – Assessment of behavioural differences in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period has been excluded. Average distance (a), average speed (b) and distance travelled at different speed thresholds (c) were plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements $>$5mm/s are considered bursting, and $<$1 mm/s are considered drifting. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. In (c), data points represent the mean. Results are for 23 (control), 25 (DSS 330 mg/L), 21 (CIT 0.75 mg/L and DSS 330 mg/L + CIT 0.75 mg/L), 18 (CIT 1.5 mg/L), and 19 (DSS 330 mg/L + CIT 1.5 mg/L) larvae per group.

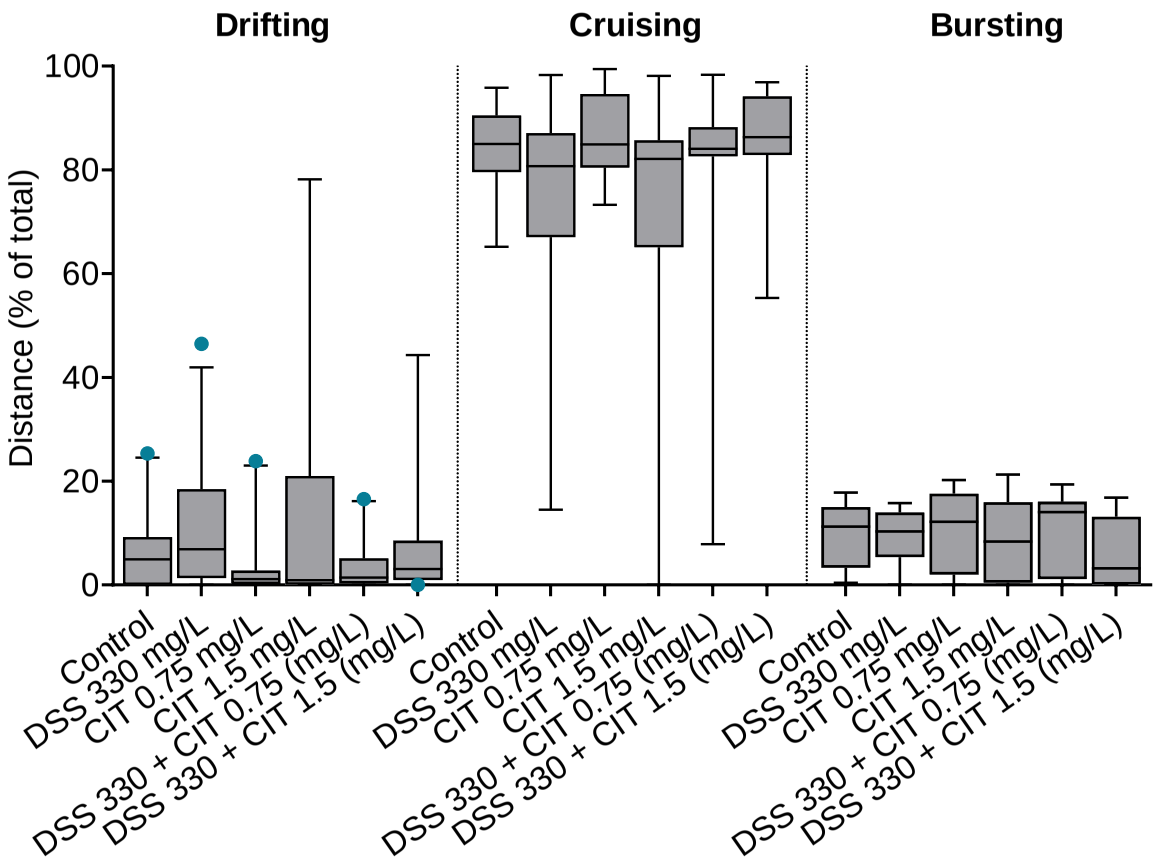
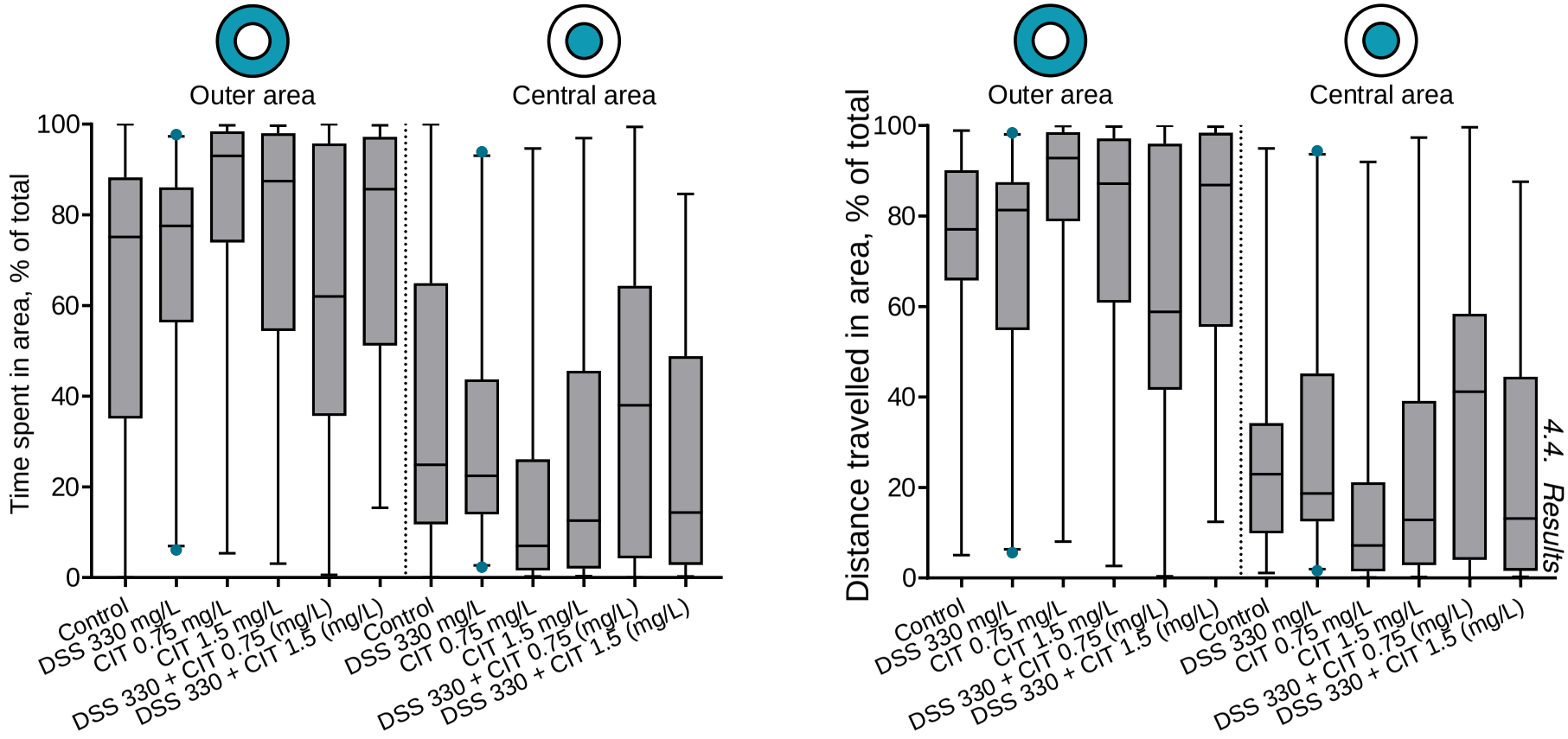


Figure 4.5 – Assessment of speed preference in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to citalopram alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-sec intervals throughout a 50-min session. The initial 10-min dark acclimation period has been excluded. Distance travelled at different speed thresholds was plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. Results are for 23 (control), 25 (DSS 330 mg/L), 21 (CIT 0.75 mg/L and DSS 330 mg/L + CIT 0.75 mg/L), 18 (CIT 1.5 mg/L), and 19 (DSS 330 mg/L + CIT 1.5 mg/L) larvae per group.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well as a percent of total distance travelled

Figure 4.6 – Assessment of thigmotaxis in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period has been excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. Results are for 23 (control), 25 (DSS 330 mg/L), 21 (CIT 0.75 mg/L and DSS 330 mg/L + CIT 0.75 mg/L), 18 (CIT 1.5 mg/L), and 19 (DSS 330 mg/L + CIT 1.5 mg/L) larvae per group.

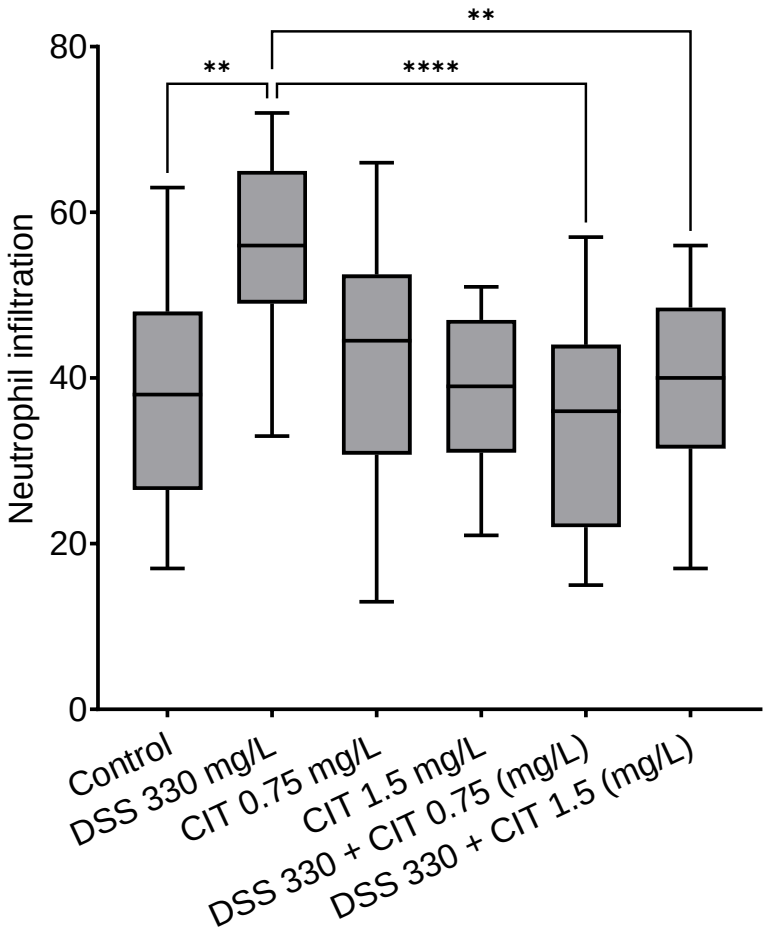


Figure 4.7 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (as demarcated in Fig. 2.4) in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. Results are for 13 (control), 15 (DSS 330 mg/L and DSS 330 mg/L + CIT 0.75 mg/L), 14 (CIT 0.75 mg/L), 11 (CIT 1.5 mg/L), and 17 (DSS 330 mg/L + CIT 1.5 mg/L) larvae per group.

4.4.2 Behaviour and immune profile of zebrafish larvae co-exposed to Dextran Sulfate Sodium (DSS) and Trazodone for 10 days

A range-finding experiment was conducted using the concentrations of trazodone determined through a literature review. At 72 hpf, after hatching, 10 Tg(MPx GFP)i114 zebrafish larvae were loaded into each well of 6-well plates containing 5 concentrations of trazodone hydrochloride (0.21875, 0.4375, 0.875, 1.75, and 3.5 mg/L) in recirculating system water, along with a water-only control. A total of 60 larvae were used,

with 10 larvae per exposure group. Zebrafish larvae were exposed for 10 days and maintained as described in the methods section.

After 4 days of exposure, the majority of larvae in the 3.5 mg/L group were non-responsive and the larvae were culled. Exposures to trazodone at 0.875 and 1.75 mg/L led to high mortality rates and larvae were terminated at 12 and 11 dpf, respectively (Fig. 4.8). In the control group and in larvae exposed to concentrations up to 0.4375 mg/L, the mortality rates observed were under 30%. As previously mentioned, on basis of baseline control data, a 30% mortality rate after the beginning of external feeding is considered to be within the limits of natural mortality. This is due to some larvae failing to initiate external feeding after depletion of the yolk sac.

Taking into consideration the mortality rates seen in larvae exposed to trazodone at or above 0.875 mg/mL, coupled with the sharp decrease in concentrations leading to higher mortality rates with longer exposures observed in the literature (306.2 mg/L for 90 minutes exposure and 40.83 mg/L for 48 hours exposure), and the reported hepatotoxicity at 16.332 mg/L (Huang et al., 2018), 0.04 and 0.2 mg/L of trazodone were selected for co-exposure with DSS. These concentrations are lower than our lower tested concentration in the range-finding experiment and 80 to 400 times lower than concentrations reported to cause hepatotoxicity.

Tg(MPx GFP)i114 zebrafish were exposed for 10 days to water control, 330 mg/L DSS alone, and 0.04 and 0.2 mg/L trazodone alone and in combination with 330 mg/L DSS. The study was carried out in 2 independent experiments with a total of 28 larvae per exposure group. At 72 hpf, after hatching, 7 larvae were loaded into each well of 6-well plates containing the selected concentrations in recirculating system water, along with a water-only control. A total of 168 larvae were used, with 28 larvae per exposure group. Zebrafish larvae were exposed for 10 days and maintained as described in the methods section.

The majority of groups displayed mortality rates under 30%, comparable to mortality in the control group (18%) and considered natural and acceptable after initiation of external feeding (Fig. 4.9). However, co-exposure to 330 mg/L DSS and 0.2 mg/L trazodone resulted in increased mortality from 12 to 13 dpf (from 29% to 61%).

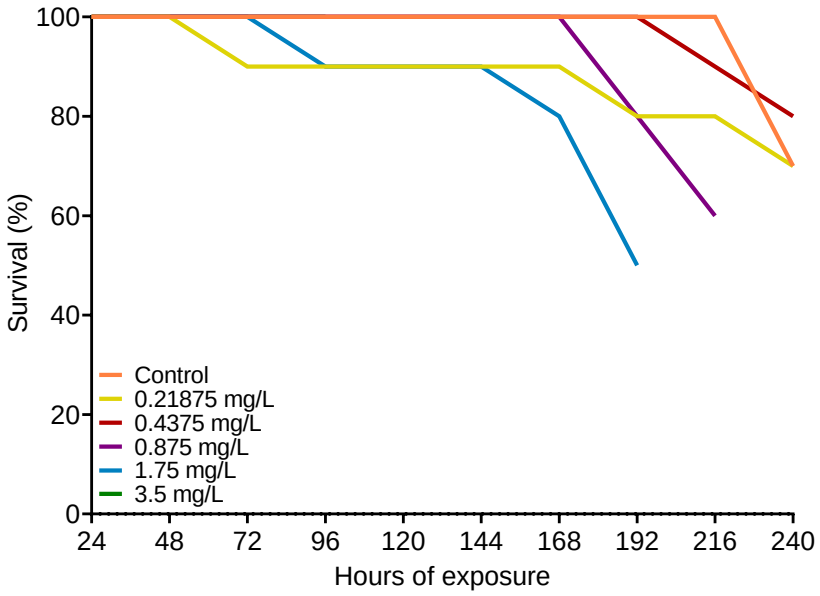


Figure 4.8 – Mortality of zebrafish after 10-day exposure to antidepressant citalopram alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Results are for 10 larvae per group. Exposure to concentrations up to 0.4375 mg/L resulted in mortality rates under 30%.

DSS and trazodone alone or in combination did not generally affect phototaxis at the selected concentrations (Fig. 4.10, Table 4.5). However, larvae exposed to 0.2 mg/L trazodone in combination with DSS displayed higher levels of activity, particularly in the last 10 minutes recorded. This peak in the last 10 minutes was observed across all plates.

Trazodone and DSS, alone or in combination, did not alter the total distance travelled or average speed of larvae (Fig. 4.11) or significantly change the distribution of movement at different speeds (Fig. 4.12). Like with citalopram, some groups displayed increased variability.

However, time spent in the central area of the well was significantly lower when compared to controls in larvae exposed to 0.2 mg/L trazodone, with or without DSS (4.13). While DSS-exposed larvae also showed a preference for spending time in the outer area of the well when compared to controls, this difference was not significant (p=0.1128). However, this response is consistent with the preference observed in previous experiments with 13 dpf larvae exposed to DSS.

Trazodone alone had no effect in the number of neutrophils present in the gut of

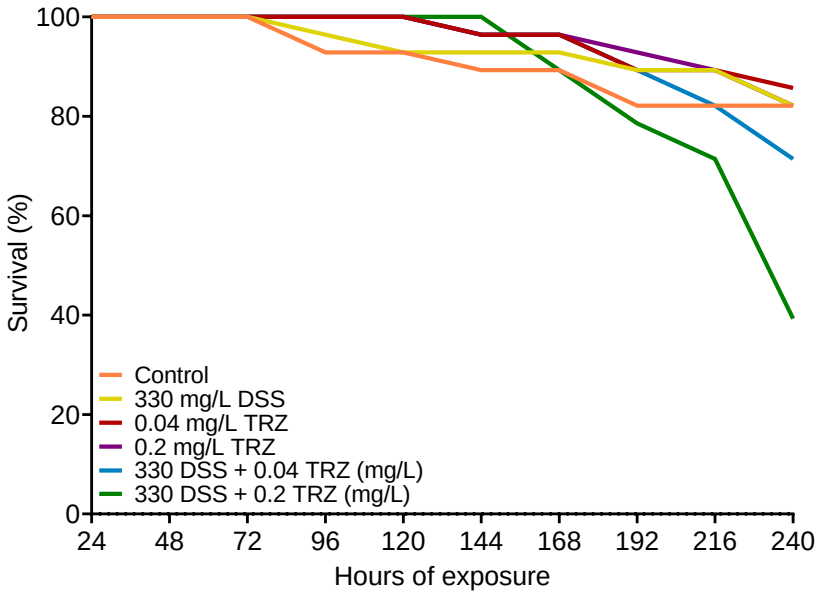
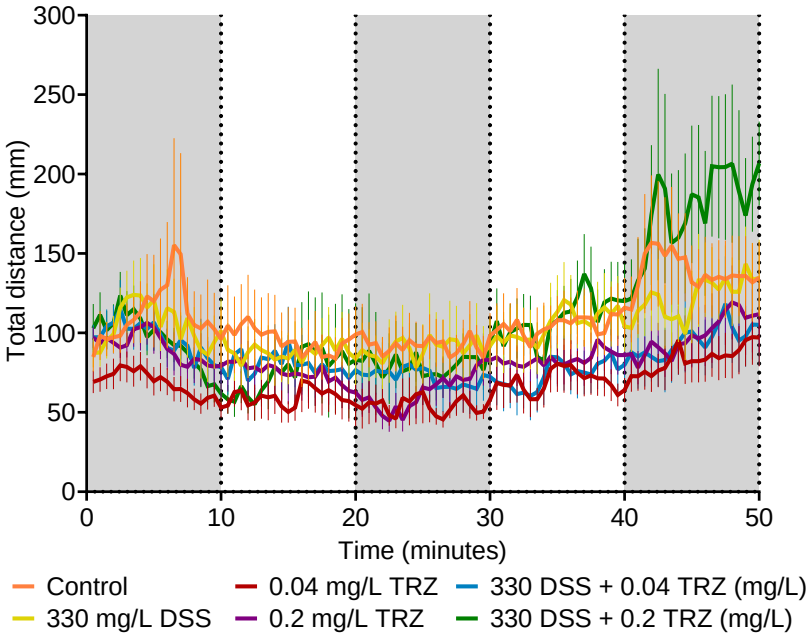


Figure 4.9 – Mortality of zebrafish after 10-day exposure to antidepressant trazodone alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Results are for 28 larvae per group. All exposure groups except DSS with 0.2 mg/L trazodone (TRZ) displayed mortality rates under 30%.

exposed larvae when compared to controls (Fig. 4.14). While DSS, as with previous experiments, significantly increased the number of neutrophils in the gut. While co-exposure with 0.04 mg/L did not recover the effect of DSS, with levels remaining the same as larvae exposed to DSS alone, 0.2 mg/L trazodone lowered the levels of neutrophils in the gut to numbers comparable with controls and significantly lower than in DSS-exposed larvae.

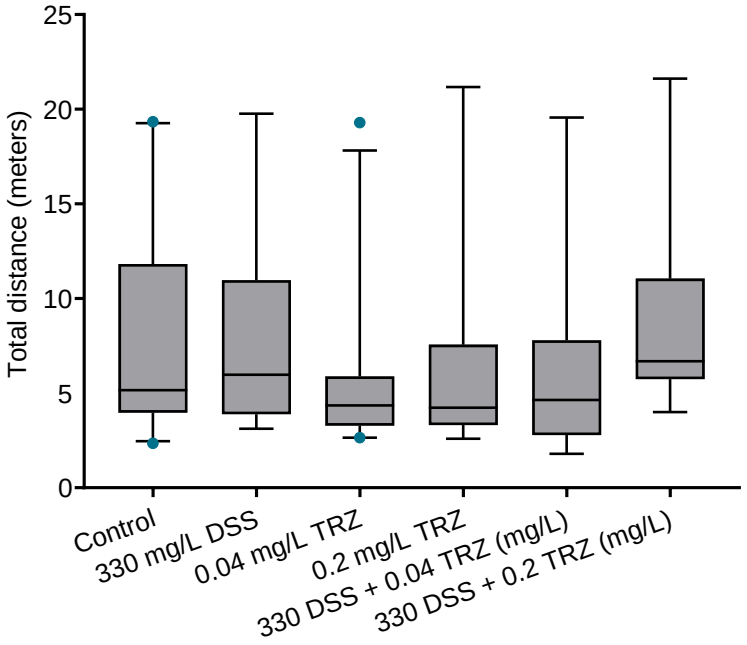


(a) Mean distance travelled by larvae under alternating dark and light conditions

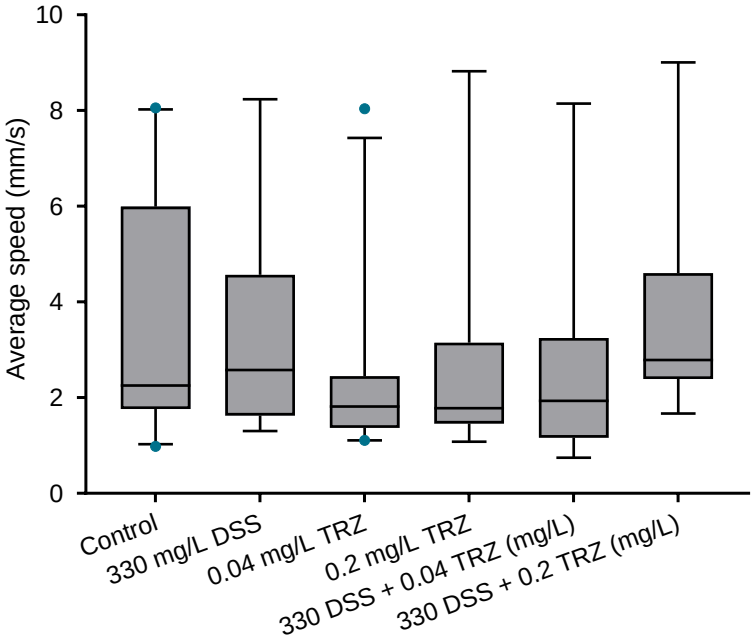
Figure 4.10 – Effect of alternating light–dark periods on locomotion in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant trazodone alone or in combination with DSS. Free swimming hatched larvae were introduced to the dilutions from 3 dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. The statistical methods applied are outlined in the methods section (2.3.8). Grey areas signify dark conditions. Data are presented as mean distance moved (in mm) in 30-second intervals throughout a 50-minute session. Results are for 23 (control), 18 (DSS 330 mg/L and TRZ 0.2 mg/L), 22 (TRZ 0.04 mg/L), 16 (DSS 330 mg/L + TRZ 0.04 mg/L), and 12 (DSS 330 mg/L + TRZ 0.2 mg/L) larvae per group.

Table 4.5 – Number of time points with statistically significant difference compared to control for data in Fig. 4.10. Data represent the response to alternating light-dark periods on locomotion after 48h of exposure to increasing doses of DSS. The statistical methods applied are outlined in the methods section (2.3.8). Results are for 23 (control), 18 (DSS 330 mg/L and TRZ 0.2 mg/L), 22 (TRZ 0.04 mg/L), 16 (DSS 330 mg/L + TRZ 0.04 mg/L), and 12 (DSS 330 mg/L + TRZ 0.2 mg/L) larvae per group.

	Control *vs.* 330 mg/L DSS	Control *vs.* 0.04 mg/L TRZ	Control *vs.* 0.2 mg/L TRZ	Control *vs.* 330 DSS + 0.04 TRZ (mg/L)	Control *vs.* 330 DSS + 0.2 TRZ (mg/L)	330 mg/L DSS *vs.* 330 DSS + 0.04 TRZ (mg/L)	330 mg/L DSS *vs.* 330 DSS + 0.2 TRZ (mg/L)
Time points with statistically significant difference *vs.*control	3	0	0	0	0	0	7



(a) Total distance travelled by larvae (meters)



(b) Average speed of larvae (mm/s)

Figure 4.11 – Assessment of behavioural differences in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant trazodone alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period has been excluded. Average distance (a) and average speed (b) were plotted. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. Results are for 23 (control), 18 (DSS 330 mg/L and TRZ 0.2 mg/L), 22 (TRZ 0.04 mg/L), 16 (DSS 330 mg/L + TRZ 0.04 mg/L), and 12 (DSS 330 mg/L + TRZ 0.2 mg/L) larvae per group.

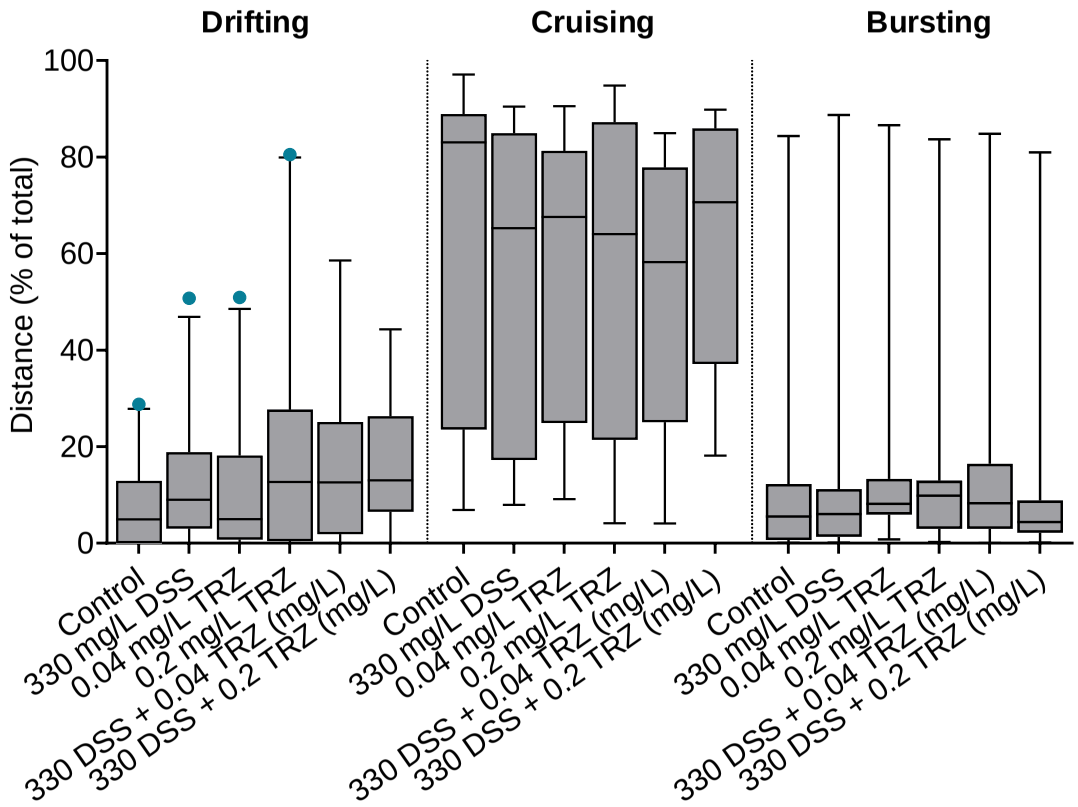
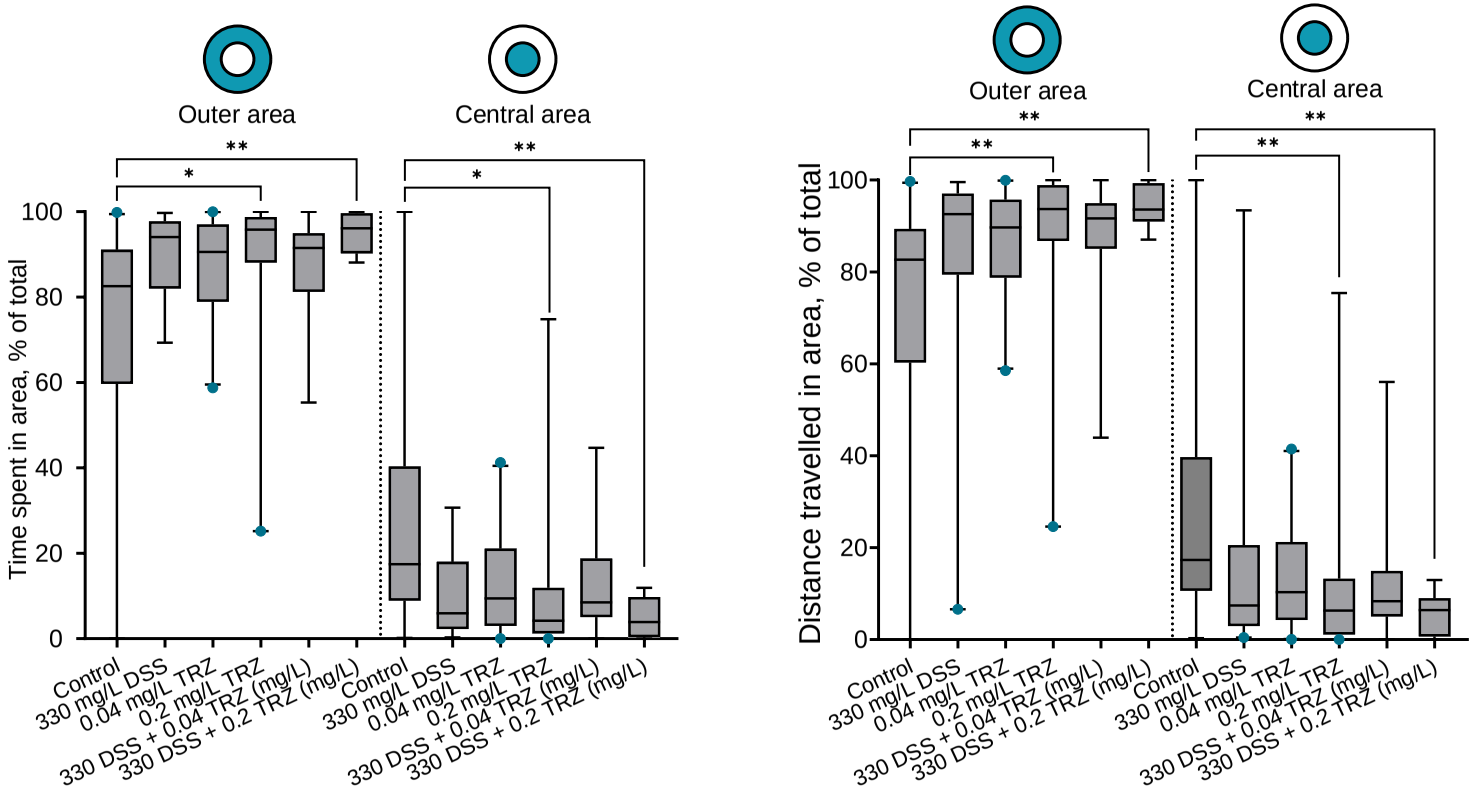


Figure 4.12 – Assessment of speed preference in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant trazodone alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period has been excluded. Distance travelled at different speed thresholds was plotted. Movements at speeds between 1-5 mm/s are normal cruising speeds, while movements >5mm/s are considered bursting, and <1 mm/s are considered drifting. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. Results are for 23 (control), 18 (DSS 330 mg/L and TRZ 0.2 mg/L), 22 (TRZ 0.04 mg/L), 16 (DSS 330 mg/L + TRZ 0.04 mg/L), and 12 (DSS 330 mg/L + TRZ 0.2 mg/L) larvae per group.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well

Figure 4.13 – Assessment of behavioural differences in Tg(MPx GFP)i114 larval zebrafish after 10-day exposure to antidepressant trazodone alone or in combination with DSS. Larvae were introduced to the dilutions from 3 dpf. Data were obtained in 30-second intervals throughout a 50-minute session. The initial 10-minute dark acclimation period has been excluded. Time spent (a) and distance moved (b) by larvae in the outer and central area of the well were plotted. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. Results are for 23 (control), 18 (DSS 330 mg/L and trazodone 0.2 mg/L), 22 (trazodone 0.04 mg/L), 16 (DSS 330 mg/L + trazodone 0.04 mg/L), and 12 (DSS 330 mg/L + trazodone 0.2 mg/L) larvae per group.

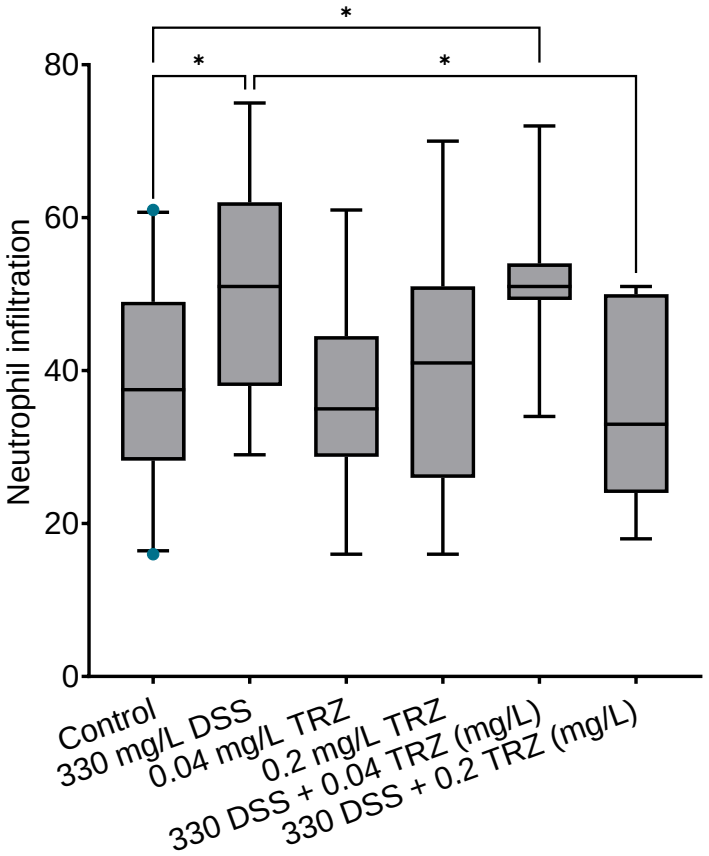


Figure 4.14 – Quantification of infiltrating neutrophils in the mid and posterior section of the gut (as demarcated in Fig. 2.4) in Tg(MPx GFP)i114 larval zebrafish after 48h of exposure to increasing doses of DSS. Larvae were introduced to the dilutions from 3 dpf. Data are presented as median with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Dots represent outliers. Results are for 23 (control), 18 (DSS 330 mg/L and TRZ 0.2 mg/L), 22 (TRZ 0.04 mg/L), 16 (DSS 330 mg/L + TRZ 0.04 mg/L), and 12 (DSS 330 mg/L + TRZ 0.2 mg/L) larvae per group.

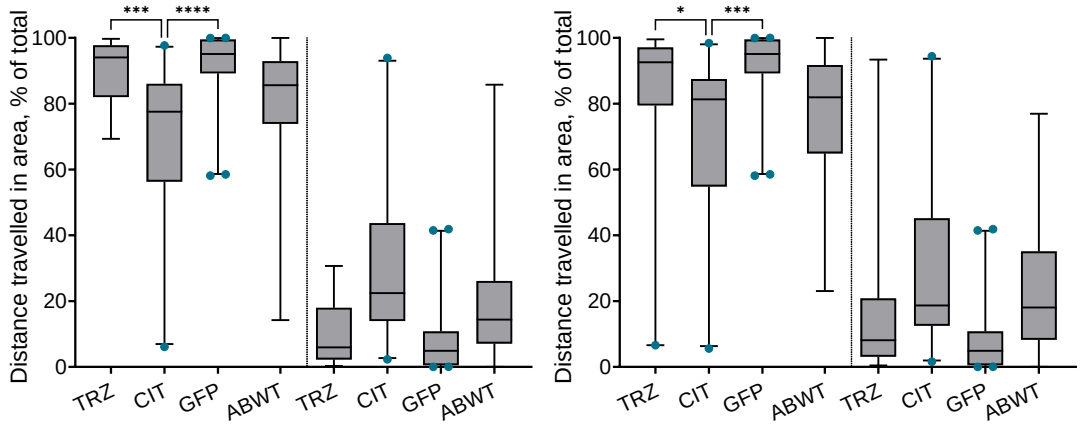
4.4.3 Comparison of DSS effects on thigmotaxis across all experiments

The set of experiments carried out in this chapter has shed light on the increased variability observed in the outcomes of thigmotaxis endpoints, bringing into question the reliability of these findings in larvae exposed to DSS.

The impact of 0.33 mg/mL DSS on zebrafish larvae exposed for 10 days was systematically analyzed across all experimental setups and subsequently subjected to Anova one-way multiple comparison tests (Fig. 4.15).

While no statistically significant difference was observed in the majority of exper-

iments, it is worth noting that the variability in the citalopram experiment stood out, resulting in outcomes significantly different from those of the Trazodone experiment and the experiments detailed in Section 2.4.4.2. While this raises concerns regarding the validity of the citalopram findings, it also bolsters confidence in the outcomes associated with Trazodone.



(a) Time spent by larvae in the central and outer area of the well as a percent of total observed time

(b) Distance travelled by larvae in the central and outer area of the well

Figure 4.15 – Comparison of behavioural differences in larval zebrafish exposed to 0.33 mg/mL DSS across 4 distinct exper- iments. Data are presented as median values with 5-95 percentile error bars. The statistical methods applied are outlined in the methods section (2.3.8). Thigmotaxis did not significantly vary in controls across these experiments.

4.5 Discussion

In our experiments described in Chapter 2, the behavioural disruption induced by DSS was consistently observed in short-term experiments (48 hours exposure) using 3-to-5 dpf larvae. However, the reproducibility of this disruption decreased with longer exposures (10 days) to lower DSS concentrations and in 13 dpf larvae. In this chapter, zebrafish larvae exposed to 330 mg/L DSS for 10 days did not show any disruption in response to dark/light stimuli and overall locomotion and speed. Regarding thigmotaxis, in the trazodone experiment, exposure to 330 mg/L DSS led to an increased preference for the outer area of the well, although not significantly in this case. In the citalopram experiment, no change in thigmotaxis was observed in the group exposed to DSS. However, as noted in the results, time and distance spent in the central and outer areas of the well display higher variability than other endpoints and than previous

experiments. The causes of these higher variability levels are unclear.

While exposure to citalopram alone or in combination with DSS did not significantly alter the behaviour of zebrafish larvae, there was an unexplained increase in movement after 20 minutes of observation in most experimental plates. Contrastingly, a decrease in locomotor activity in zebrafish has been reported in the literature (Kulikova et al., 2021; Hong et al., 2021; Bachour et al., 2020; Steele et al., 2018b). However, published studies were performed either with larvae up to 144 hpf, where dark swimming activity was decreased, or in juveniles and adults, where overall locomotor activity was decreased. As the zebrafish larvae in this study were between these ages, direct comparison with available literature is difficult. The behaviour of zebrafish, whether they are untreated or drug-treated, has been extensively documented to exhibit a significant level of individual variation (Tanoue et al., 2019; Huerta et al., 2016; Margiotta-Casaluci et al., 2014). This underscores the necessity for additional trials to confirm the reproducibility of these findings.

The behaviour outcomes obtained with trazodone, on the other hand, were clear. While distance and speed were not altered in larvae exposed to trazodone alone or with DSS, thigmotaxis was significantly altered. As seen with larvae exposed to 330 mg/L DSS alone, larvae exposed to 0.2 mg/L trazodone displayed classic thigmotactic behaviour, preferring the outer area of the well versus the central area, which is generally considered a measure of zebrafish anxiety. While the citalopram experiment displayed increased variability and significantly different thigmotaxis outcomes for larvae exposed to DSS alone, the results with DSS in the trazodone experiment matched the previously observed, providing increased confidence in the results seen for thigmotaxis in this experiment.

The similar response to DSS and trazodone in combination and DSS alone could simply be explained by a lack of effect of trazodone in rescuing the behaviour alteration caused by DSS alone. However, curiously, trazodone alone also significantly increased thigmotaxis. Data on the effects of trazodone in zebrafish are very scarce, and further experiments could help clarify if this is a reproducible effect or a case of high individual variation between larvae.

While the antidepressants tested were expected to lead to a reduction of the anxiety-like behaviour induced by DSS, it is worth noting that the anti-inflammatory dexamethasone also did not rescue behaviour alterations induced by DSS in Chapter 2. This could mean that while antidepressants might have anti-inflammatory action similar to anti-inflammatory drugs, the nuanced behaviour-altering effect of dexamethasone, citalopram, and trazodone was not captured in these experiments.

It is of particular interest that the infiltration of neutrophils in response to both antidepressants indicates the possibility of an anti-inflammatory effect. As consistently seen in our previous experiments, DSS leads to an increase in the number of neutrophils in the mid and posterior gut of zebrafish larvae. Both concentrations of citalopram and the highest concentration of trazodone rescued this phenotype, significantly reducing the levels of neutrophils in the gut to levels comparable to controls. In Chapter 2, it was demonstrated that co-exposure with DSS and anti-inflammatory dexamethasone also fully recovered this effect of DSS, with neutrophil infiltration reducing to levels that are comparable to those of the control group, further pointing to the potential of these antidepressants to act as anti-inflammatory agents. Considering that patients with MDD have been shown to express higher neutrophil-to-lymphocyte ratios than healthy individuals (Darko et al., 1988; Maes et al., 1994; Irwin et al., 1987; Kronfol and House, 1989; McAdams and Leonard, 1993), while MDD patients taking SSRIs have normalized NLRs (Demircan et al., 2016), this represents new evidence for the role of neutrophils and inflammation in the pathophysiology of depression. Regardless, neutrophil recruitment represents merely a fraction of the overall inflammatory response, and a more comprehensive investigation of the effects of these drugs on the wider immune response is necessary.

It is important to note that peripheral anti-inflammatory actions may not necessarily indicate an anti-inflammatory action in the brain, as microglia, the primary cells involved in inflammatory processes in the brain, function differently from immune system cells that mediate the anti-inflammatory effects of antidepressants in the periphery (Tynan et al., 2012; Graeber, 2010; Wake et al., 2009). However, recent studies have shown that some antidepressants, including fluoxetine, paroxetine, and sertraline, can affect

certain aspects of the microglial inflammatory response by modulating the ability of murine microglia to produce the cytokine TNF-α and the free radical NO (Hashioka et al., 2007, 2009; Horikawa et al., 2010; Hwang et al., 2008; Ha et al., 2006).

In our drug-gene interaction pathway enrichment analysis, we observed that six antidepressants from various classes interacted with genes associated with neutrophil degranulation. However, citalopram and trazodone did not interact with this pathway. Citalopram only interacted with two pathways after 6 hours of exposure, and none after 24 hours, whereas trazodone interacted with 17 pathways after 6 hours and 12 pathways after 24 hours. Despite the evidence from our zebrafish imaging experiments suggesting that both trazodone and citalopram have high anti-inflammatory effects, our pathway enrichment analysis revealed that trazodone ranked high and citalopram ranked low in terms of immunomodulatory potential. This raises doubts about the ability of perturbagen databases and pathway enrichment to predict the anti-inflammatory potential of these drugs and further experimental validation of predicted drug-gene interaction enriched pathways remains crucial. It is important to consider that our computational analysis was focused on REACTOME molecular pathways involved in innate and adaptive immunity, as well as cytokine signalling. However, it is possible to hypothesize that other non-immune pathways play an important role in the mediation of inflammatory responses. Hence, future studies should expand the assessment of the transcriptomic signature of anti-depressants to include non-immune pathways. Due to technical and resource constraints, RNAseq analysis of exposed larvae was planned but not performed. Investigating the differential expression patterns in zebrafish larvae exposed to these drugs may offer additional insights into the *in vivo* translational value and reproducibility of the cell-based data used in Chapter 3, and facilitate the exploration of other relevant inflammation-related pathways that may link chemical-induced inflammation to behavioural effects.

4.6 Conclusion

Although the behavioural outcomes induced by DSS were in line with previous experiments, the impact of trazodone and citalopram on zebrafish behaviour remains

uncertain. To validate the observed results and rule out the effect of inter-individual variability, additional trials are necessary.

While this study did not directly validate the predictions of our drug-gene interaction pathway enrichment analysis, both citalopram and trazodone demonstrated clear anti-inflammatory effects by rescuing the increased neutrophil infiltration to the gut oz zebrafish exposed to DSS. While neutrophil recruitment represents merely a fraction of the overall inflammatory response, and further investigation of the effects of these drugs on the wider immune and non-immune response is necessary, this represents new evidence for the direct immunomodulatory activity of antidepressants.

Additionally, conducting further trials with antidepressants ranked both at the top and bottom of inflammatory potential, as identified in Chapter 3, could elucidate whether different antidepressants indeed possess the predicted distinct anti-inflammatory capabilities. The exploration of other immune markers implicated in depression, such as TNF-alpha and IL-6 — recognized as predictors of antidepressant effectiveness — or others like IL-17A, which are suggested as markers of therapy resistance, could provide additional evidence for the anti-inflammatory effects of antidepressants. The use of transgenic zebrafish with fluorescence markers for these compounds, such as the ones employed in the experiments outlined in this thesis, could provide insights into these hypotheses. Moreover, as discussed in Chapter 2, examining the differential expression patterns in zebrafish larvae exposed to these drugs through transcriptomics may offer additional insights into the in vivo effects and elucidate relevant pathways involved.

4.7 References

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Chapter 5

General conclusions

This chapter summarizes the main findings from the studies in this thesis, the contributions made to the field of psychoimmunology and inflammation biology, and makes recommendations for future research.

5.1 Main findings

The main goal of this research project was to investigate the relationship between peripheral inflammation and the manifestation of behavioural alterations, utilizing a combination of experimental methods based on the zebrafish model (*i.e.* behavioural phenotyping and immune *in vivo* imaging) and computational methods that leverage the potential of human-relevant large-scale omics datasets (*i.e.* assessment of drug immunomodulatory signature at transcriptomic level). In Chapter 1, we explored the complex relationship between the brain, immune system, and inflammation. We detailed the various neuropsychiatric disorders that have been linked to peripheral immune and inflammatory responses, with a focus on major depressive disorder (MDD). To understand the complex interplay of immune responses and neurotransmitter metabolism, we developed an evidence map based on existing literature. The relationship between peripheral inflammation and behavioural alterations has an important clinical and epidemiological relevance, as many life-style factors have been demonstrated to induce inflammation in human populations, including pollution, stress, and high-fat diet. Therefore, there is an urgent need to understand to what extent such inflammation contributes to the etiology of behavioural disorders, and to characterize the complex

interplay of mechanisms underlying this complex biological phenomenon.

Establishing causal relationships between stressors and biological effects directly in humans is extremely challenging due to the numerous confounding factors that can affect the interpretation of human epidemiological data. For this reason, non-human model species are needed to investigate the biological processes of interest under controlled conditions. This is particularly true for animal behaviour, which has evolved to ensure an effective adaptation to environmental conditions. Small rodent species have played and still play a pivotal role in the advancement of our understanding of the relationship between peripheral immunity and brain functions. In this project, we hypothesized that many relevant components of such relationship could be recapitulated also in zebrafish, with significant potential benefits in terms of 3Rs. In line with this vision, the first experimental phase of this project, described in Chapter 2, focused on the characterization of the behavioural responses triggered by exposure to pro-inflammatory stimuli. The aim of this work was to identify a suitable and reproducible zebrafish larval model of inflammation-induced behavioural alteration. After testing five different pro-inflammatory conditions (*i.e.* trinitrobenzene sulfonic acid (TNBS), dextran sodium sulfate (DSS), Copper Sulphate, lipopolysaccharide (LPS), high-fat diet) well characterised in mammalian models, the results indicated that DSS-induced inflammation is associated behavioural alterations in zebrafish as it is in rodent models. Therefore, the DSS-induced inflammation model was selected for the second phase of the project, which involved the co-treatment of inflamed larvae with antidepressant drugs. Despite its potential, it is important to highlight that whereas the behavioural disruption was consistently observed in short-term experiments (48 hours exposure) using 3-to-5 days post-fertilization (dpf) larvae, the reproducibility of this disruption decreased with longer exposures (10 days) to lower DSS concentrations and in 13 dpf larvae (see Section 5.3 for a more detailed discussion of such limitation). The other tested pro-inflammatory treatments were deemed to be unsuitable for co-exposure experiments due to a range of factors, including lack of simultaneous effect on both inflammatory responses and behaviour, incompatibility with drug co-exposure to do strong oxidising properties (TNBS), lack of statistically significant effects (high-fat diet, LPS), techni-

cal difficulties with the imaging process (copper sulphate). Once a suitable model of inflammation-induced behavioural alterations was identified, the second phase of the project aimed at characterising the potential of 20 different antidepressants to modulate immune and inflammatory responses using computational methods (Chapter 3), and at testing the ability of some of these antidepressants to rescue inflammation-induced behavioural disruption *in vivo* using the inflammation model developed in the first phase of the project (Chapter 4).

Based on the evaluation of transcriptomic data generated using 16 different human cells lines with standardized protocols (with all drugs tested in the same conditions, doses, and time points), and the enrichment of functional pathways, in Chapter 3 we predicted that different classes of antidepressants interact to a different degree with pathways involved in innate and adaptive immunity and cytokine signalling. This immunomodulatory action varied between antidepressants and even within the same class (*e.g.* selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs)). Inflammatory markers that have been suggested in the literature as predictors of antidepressant effectiveness (such as tumor necrosis factor (TNF) alpha and interleukin (IL)-6) or markers of therapy resistance (*e.g.* IL-17A) were correctly predicted in our computational method to be targets of antidepressant action. Some still relatively unknown inflammatory markers of depression (*e.g.* IL-20) were also highlighted, and some antidepressant classes (*e.g.* serotonin antagonist and reuptake inhibitors (SARIs), in particular trazodone) were identified as candidates with high immunomodulatory potential, suggesting a potential role for these drugs in the treatment of depression.

To validate these results, in Chapter 4 we describe a set of *in vivo* experiments carried out with trazodone and citalopram, two antidepressants predicted to have, respectively, high and low immunomodulatory potential. These experiments were designed to test the ability of these compounds to rescue the inflammatory and behavioural responses induced by DSS. Contrary to the prediction, both antidepressants, not just trazodone, exhibited significant anti-inflammatory effects in zebrafish exposed to DSS. This was evident from their ability to rescue the increased infiltration of neutrophils in

the intestine induced by DSS exposure, suggesting that the anti-inflammatory properties of these drugs may play a role in mediating their therapeutic effects. Despite the observed effect on neutrophil recruitment and infiltration, the impact of trazodone and citalopram on zebrafish behaviour was difficult to interpret. This is likely due to a combination of factors, that include the complexity of testing the effects of chemical co-exposures on behaviour, and the unexpected limited degree of reproducibility of DSS-induced behavioural responses in older larvae.

5.2 Scientific contribution

The research presented in this PhD thesis makes several important contributions to the field of psychoimmunology and antidepressant pharmacology.

The evidence map developed in Chapter 1 represents a new visual tool that can be used for identifying gaps in knowledge in the complex relationship between immune responses and neurotransmitter metabolism, highlighting areas that require further research. Gaining a better understanding of the components involved in inflammation and depression, will aid in the development of more effective treatments and improving outcomes for those affected by these conditions.

The study of DSS-induced inflammation in zebrafish represents a novel approach to investigating the relationship between inflammation and behaviour. Our work suggests that the zebrafish model could serve as a complementary model to investigate this relationship and serve as a stepping stone for future investigations aimed at elucidating the precise underlying mechanisms. The proposed zebrafish model of inflammation-induced behavioural disruption serves as a preliminary step towards the development of further models, in which inflammation could be induced through genetic manipulations instead of chemical exposure. Such an inflammation model would prove highly valuable in designing screening programs aimed at identifying compounds that can rescue inflammation-induced behaviour before initiating pre-clinical work using higher vertebrate models. However, it's worth noting that chemical-induced inflammation also has significant public health implications. Therefore, the DSS model presented in this thesis will aid in enhancing our comprehension of chemical safety from a

neuro-immune perspective.

Our research in Chapter 3 provides valuable insights into the complex interaction between antidepressants and the immune system, by addressing the knowledge gap in our understanding of the immunomodulatory potential of antidepressants. Our results revealed that different classes of antidepressants interact with various immune pathways, and even within the same class, immunomodulatory action can vary, emphasizing the need for personalized medicine considerations in developing more effective treatment strategies for depression and other behavioural disorders where inflammation is a contributing factor.

Our work also demonstrates the potential of drug-induced perturbation databases such as the Library of Integrated Network-Based Cellular Signatures (LINCS) connectivity map for computational drug discovery approaches. Using *in silico* methods, we were able to characterise the comparative immunomodulatory potential of 20 different antidepressants at a transcriptomic level. To our knowledge, this is one of the first attempts that apply a comparative transcriptomic perspective to a wide range of antidepressants. These results can be expanded in the future by including non-immune pathways that also play a significant role in the mediation of inflammatory responses. These findings could enhance our ability to identify compounds that have a beneficial impact on the neuro-immuno-pharmacological profile.

5.3 Research limitations and future perspectives

While the findings of this thesis advance our current understanding of the relationship between peripheral inflammation and behaviour in the zebrafish model, it is important to acknowledge the limitations of this research. In this section, we will discuss such limitations and the potential solutions that could be implemented in future research to overcome them.

The first set of limitations concerns the zebrafish experimental phases. The high degree of variability typically observed in animal behaviour, including rodent studies, represented one of the rationales for the use of the zebrafish model in the present project. The behaviour of 5 dpf zebrafish larvae is well characterised, and the regular

patterns of response to stimuli such as light exposure and wavelengths are commonly used in phenotypic drug discovery programmes (Kokel and Peterson, 2008; Kokel et al., 2010; Kokel and Peterson, 2011). While in the present research, zebrafish behavioural responses to DSS did display a high level of reproducibility, one of the aims of this project was to investigate the behavioural effects of lower non-pathological inflammation intensity, going beyond potential sickness behaviours. To do so, we lowered the exposure concentrations of DSS (approximately 15 times lower than the concentrations used in the published literature) and extended exposure times from 2 days to 10 days, in order to generate a physiological setting that was closer to the longer-term clinical scenarios of interest (*i.e.* chronic low-grade inflammation). 13 dpf larvae were considered suitable to perform both *in vivo* imaging and behavioural phenotyping, thanks to the acceptable level of transparency, which decreases rapidly after that time point. In the present study, while we observed consistent behavioural disruption induced by DSS in 3-to-5 dpf larvae during short-term (48-hour) exposure, we also noted a decrease in the reproducibility of this disruption during longer (10-day) exposures to lower DSS concentrations in 13 dpf larvae, which represented a challenge in terms of data interpretation and reproducibility of results. Due to 3Rs implications, 4-5 dpf larvae are the most popular life stage in behavioural pharmacology research. As there has been a limited amount of research conducted on older zebrafish larvae, a comprehensive assessment of the development of relevant behavioural responses is currently lacking (Petersen et al., 2022). Nonetheless, previously published research supports our observations. For example, Gould (2022) also observed a change in the behavioural response of zebrafish to dark-light stimuli, from 5 to 28 dpf zebrafish.

The detection of glitches in behavior tracking raises potential concerns about the reliability of collected data. Video footage from all behavioral experiments underwent manual review, during which glitching wells — many of which were previously flagged as outliers during statistical analysis — were systematically removed. Upon investigating the frequency of these errors, a statistically significant relationship emerged between the occurrence of glitches and the presence of empty or deceased larvae-containing wells. While the precise mechanisms driving this correlation remain un-

clear, its likely that these glitches stem from the tracking software having a tendency to overcompensate when attempting to track movement where it isn't present, thereby inadvertently capturing water reflections. However, further investigation is needed to validate this hypothesis and to identify and address other potential confounding factors that might impact the reliability of behavior-tracking data.

Although most outliers consisted of behavior tracking glitches and were consequently eliminated, the remaining outliers were deliberately retained for analysis. While the inclusion of outliers poses a risk of distorting results and warrants cautious interpretation of findings, it remains crucial, particularly within the context of behavioral data, to encompass the entirety of behavioral diversity within the population. This entails acknowledging that certain zebrafish may exhibit greater activity levels than others, and that individual responses to exposure compounds may vary drastically.

While the use of larval and juvenile animals in toxicology and drug discovery is standard practice, as evidenced by the extensive literature cited in this thesis, the age-specific effects of antidepressants across the life cycle of fish remain unknown. The variability in response to antidepressants between juvenile and adult humans (Bylund and Reed, 2007) underscores the intricate nature of age-related responses and emphasizes the necessity for further exploration in future research. If older larvae are deemed to be a useful life stage for psycho-pharmacology investigations, a systemic characterisation of the temporal evolution of zebrafish behaviour could be a desirable area of focus of future research, as this knowledge could facilitate the design of future studies and data interpretation. Another area of improvement for the future is represented by the data analysis methods that are used to quantify zebrafish behaviour. Due to the complexity and multidimensional nature of behavioural datasets, multiparametric hierarchical clustering analysis could represent a more suitable approach for the analysis of complex datasets. This clustering method would allow for the detection of the shift in behavioural patterns beyond the indications provided by standard statistics. An example of such an approach is the data analysis pipeline used for drug discovery by Kokel and Peterson (2011), which uses fully automated platforms for analyzing the behaviour of embryonic zebrafish. Video recordings of drug-exposed zebrafish are

then transformed into numerical barcodes providing a concise and interpretable summary of the observed phenotypes for each animal. Other promising approaches for the analysis of complex behavioural datasets are represented by Bayesian modelling (Johnson et al., 2020) and the application of Machine Learning for the classification of behavioural phenotypes (Bohnslav et al., 2021; de Chaumont et al., 2019).

Still in the context of behavioural analysis experiments, utilizing thigmotaxis as a behavioural endpoint to assess alterations in behaviour has inherent limitations that require consideration. Primarily, the intricate relationship between dosage levels, exposure time and ensuing toxicity can profoundly influence thigmotactic patterns. The toxicity of the compound, whether due to a high initial dose or accumulation in the subject over time, can affect basic behaviours such as feeding and locomotion, interfering with thigmotaxis through pathways unrelated to anxiety. Furthermore, thigmotactic responses may shift over time, being potentially obscured by behaviour analysis being restricted to a 50-minute time period after 48-hour or 10-day exposure. Lastly, the inherent individual variability among experimental subjects adds a layer of complexity, as variations in sensitivity to the exposure compound can lead to distinct thigmotactic responses across the cohort. In addition to the above-outlined measures to tackle variability, selecting concentrations that do not induce toxic effects is crucial for addressing these limitations, emphasizing the importance of determining the Maximum Tolerated Concentration (MTC) and conducting pilot experiments.

Despite the high variability observed across experiments, particularly in the 10-day exposure trials, it is worth noting that behavioral endpoints such as thigmotaxis did not exhibit significant differences among control groups for the different 10-day DSS exposures. While this observation lends support to the reliability of the methodology employed, it also implies the potential influence of unaccounted factors in the study design on behavioral outcomes for DSS-exposed larvae, highlighting the need for better understanding of zebrafish behaviour.

As highlighted in 2, the isolation of zebrafish in individual wells during exposure and behavior tracking represents a departure from zebrafish's inherently social nature, and has the potential to introduce an aditional social stressor that could affect

behavioural results. While the isolation of zebrafish is standard practice in behavioral, toxicological, and drug discovery, the implications of this isolation on larval behavior and brain development remains unknown. Future technological advancements may enable behavior tracking methods that yield precise data without necessitating the isolation of zebrafish.

A further limitation of the present project was the lack of quantification of the concentration of the test chemicals in both water and whole body; hence all concentrations indicated in this work are nominal. Previous research carried out with adult fish has demonstrated that fish exposed to psychoactive drugs display a high degree of inter-individual variability in plasma concentrations of such chemicals (Huerta et al., 2016; Margiotta-Casaluci et al., 2014). This variability may have contributed to the observed behavioural variability, as discussed above. The quantification of exposure concentrations in zebrafish studies is increasingly common in toxicology studies; however, it remains rare in pharmacology research. The implementation of an analytical chemistry step (*e.g.* LC-MS/MS) in the pipeline of future research projects may elucidate the actual concentration reached in the organism and facilitate the interpretation of the phenotypic data and their variability.

It is also crucial to acknowledge and address the potential sources of bias that may have influenced the interpretation of results. The use of Zebralab software during the behavioural analysis and the deliberate blinding of exposure group information during microscopy image acquisition were essential steps in minimizing bias. However, it is important to recognize the limitations associated with the quantification of neutrophil infiltration conducted by a single individual. Despite the implemented safeguards such as blinding, replication, and predefined analysis criteria. To enhance the robustness and reliability of the study, inter-rater reliability assessments by a second analyst were planned. However, timelines were disrupted by the COVID-19 pandemic. In future experiments and data analysis, the involvement of multiple analysts who independently assess the same set of images can provide a more comprehensive evaluation of consistency and help identify and address any potential bias introduced by individual analysts, contributing to a more objective and rigorous interpretation of the data.

Inflammation was the key biological response of interest in the present project. Neutrophil and macrophage trafficking in the larvae (*e.g.* infiltration in the tissue of interest) were considered as the biological proxy for the assessment of the pro-or anti-inflammatory activity of the test compounds. While neutrophil and macrophage responses are key drivers of inflammation, it is important to consider that other aspects of the inflammatory response that can lead to behavioural changes in response to inflammation were not quantified in the present study, including cytokine signalling, the involvement of the hypothalamic-pituitary-adrenal (HPA) axis, glucocorticoid and cortisol levels, and neurotransmitters dynamics. Future research in this field could expand the assessment of inflammatory responses by developing a multi-endpoint panel able to capture the different aspects of the inflammatory response. To address this challenge, RNA sequencing experiments were planned in the initial phases of the project; however, these could not be carried out due to time and technical constraints.

Investigating the transcriptional responses in zebrafish larvae exposed to pro-inflammatory stimuli may provide additional insights into specific inflammatory markers and aid in exploring the inflammatory theory of behavioural alteration in mental health disorders like depression. Our preserved larvae samples can facilitate future studies of expression data, which could help validate the inflammatory results observed in the imaging experiments.

In the present project, inflammation was induced via exposure to exogenous stimuli (*e.g.* DSS). This choice was based on the epidemiological observation that many lifestyle factors, including chemical pollutants, can induce inflammation in human populations. However, the use of exogenous stimuli is also linked to a higher experimental variability. A complementary approach that can be used in the future would be to use gene editing methods (*e.g.* CRISPR-Cas9) to generate novel transgenic lines overexpressing pro-inflammatory genes, either systemically or in a tissue-restricted manner. This would provide more control on the induction of inflammation and would allow to consistently induce inflammation in large populations of animals, leading to a more robust quantification of the relationship between inflammatory responses and other relevant phenotypes (*e.g.* behaviour).

In addition to the limitations related to the zebrafish model, another important limitation of this project concerns the use of transcriptomic signatures for the assessment of the immunomodulatory activity of antidepressants, as described in Chapter 3. While the L1000 connectivity map database is a valuable tool for exploring the transcriptomic responses of cells to various perturbations, it comes with some known limitations. In this project, the main limitation observed was related to the low degree of reproducibility of transcriptomic responses across the different human cell lines used in the screening programme. The biological significance of this variability is currently unclear, but it suggests that transcriptomic data must be used with caution, and should be integrated with other lines of evidence generated at higher levels of biological organization. Furthermore, while the LINCS-L1000 dataset has provided valuable insights, it is also essential to acknowledge the limitations posed by its reliance on cancer cell lines. Despite their widespread availability and well-established characterization, these cell lines may not represent normal cellular physiology. Careful consideration is required to avoid interpretation bias and concerns about the applicability of findings to non-cancer contexts.

Methodologies that incorporate other omics, such as metabolomics and proteomics, as well as dynamic or longitudinal data, could help us gain a more thorough comprehension of drug mechanisms. Equally unclear is the *in vivo* translational value of the transcriptomic responses available in the Connectivity Map database. Future research could perform a systematic concordance analysis of *in vitro-in vivo* transcriptomic responses to shed light on this important knowledge gap. Experimental validation of the drug-gene interaction enriched pathways predicted in chapter 3 remains crucial. In particular, studies involving primary cells from patients with MDD, treated with antidepressants predicted to have high immunomodulatory potential, could greatly improve the understanding of the aetiology of the disease.

5.4 Conclusion

Overall, this thesis contributes to a growing body of research that explores the relationship between inflammation, behaviour and mental health disorders like depression.

The findings support the widely reported hypothesis that inflammation is associated with behavioural alterations and that antidepressant drugs may exert their effects, at least in part, through modulating immune pathways. Additionally, the zebrafish model employed in this thesis may serve as a valuable tool for investigating the relationship between inflammation and behaviour and for identifying potential therapeutic targets.

However, there are still many unanswered questions, and further studies are necessary to validate the findings presented in this work. This thesis serves as a stepping stone for future studies aimed at elucidating the precise mechanisms underlying the relationship between inflammation and behaviour, and for identifying new therapeutic targets for mental health disorders.

5.5 References

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Appendix A: Sample sizes

The sample size was determined based on the mean and standard deviation of distance traveled in the baseline experiment with zebrafish larvae reared in recirculating system water. Power was set at 80%, with a significance level of 0.05. Hypothetical decreases of 20%, 25%, and 30% in distance traveled were considered, aiming to detect effect levels between 20% and 30%. The calculations were conducted using the online calculator for Inference for Means: Comparing Two Independent Samples, available at https://www.stat.ubc.ca/ rollin/stats/ssize/n2.html. The results are presented in the table below.

Table A.1

Mean distance travelled	Standard Deviation	20% decrease	25% decrease	30% decrease
2095	711.6	1676	1571.25	1466.5
Sample size		46	29	21

The sample size for each individual experiment is provided below. The number of replicates per experiment may vary due to the specific goals of each experiment and factors such as mortality, plate setup, and egg availability.

Table A.2 – Summary of biological replicate counts per exposure group across experiments analyzing zebrafish behavior in 24-well plates. Some experiments (and corresponding plates) were distributed over two consecutive days.

Experiment	Biological replicates per exposure group	Number of 24-well plates	No. of experiments
Water vs E3 medium	36	3	1
AB WT vs trangenic lines	36	5	1
TNBS MTC (48h)	24	6	1
LPS MTC (48h)	20	5	1
LPS MTC (24h)	12	3	1
CUSO4 MTC (48h)	36	6	1
DSS MTC (48h)	24	4	1
TNBS (48h)	41	7	2
CUSO4 (48h)	48	8	2
DSS (48h)	24	4	2
DSS MTC (10 days)	4	1	1
DSS GFP (10 days)	32	8	2
DSS GFP (10 days)	24-48	4	1
DSS MCherry (10 days)	32	8	2
DSS AB WT (10 days)	24	3	1
High-fat diet + cholesterol	48	4	1
DSP MTC (10d)	10	3	1
DSS + DSP (10d)	32	8	2
Citalopram MTC (10d)	10	3	1
Citalopram + DSS (10d)	28	7	2
Trazodone MTC (10d)	10	3	1
Trazodone + DSS (10d)	28	6	2

Appendix B: Frequency of glitching in behavioural tracking

Table B.1 – Frequency of Glitching in Behavioral Tracking. The table presents the percentage of wells exhibiting glitches, calculated in relation to the total number of wells throughout the experiment. Wells containing dead larvae or empty and displaying glitches were excluded from this calculation. Additionally, the percentage of empty wells or wells with dead larvae is provided, determined in relation to the total number of wells in the entire experiment.

Experiment	% glitching wells	% wells empty or with dead larvae
Water vs E3 medium	2.78	0.00
AB WT vs trangenic lines	0.93	0.00
TNBS MTC (48h)	9.03	6.25
LPS MTC (48h)	3.33	0.00
LPS MTC (24h)	2.78	0.00
CUSO4 MTC (48h)	7.64	41.67
DSS MTC (48h)	7.29	40.63
TNBS (48h)	0.00	0.00
CUSO4 (48h)	0.00	0.00
DSS (48h)	3.13	5.21
DSS MTC (10 days)	0.00	8.33
DSS GFP (10 days)	4.17	13.02
DSS GFP (10 days)	1.04	7.29
DSS MCherry (10 days)	6.77	21.88
DSS AB WT (10 days)	8.33	33.33
High-fat diet + cholesterol	1.04	0.00
DSS + DSP (10d)	1.56	12.50
Citalopram + DSS (10d)	13.10	10.71
Trazodone + DSS (10d)	10.90	28.21

The data in table B.1 was analyzed using the Pearson correlation coefficient (Fig. B.1). The correlation analysis reveals a statistically significant (two-tailed p-value = 0.0049) relationship between the percentage of wells exhibiting glitches and the percentage of empty wells or wells containing dead larvae. The Pearson correlation coefficient of 0.6169 indicates a moderately strong positive linear relationship between the two variables. The regression analysis for this data indicates a positive relationship with an R squared value of 0.3806, indicating that the variance in glitch occurrence could be explained by the percentage of empty or dead larvae-containing wells. The observed correlation might be influenced by the software potentially overcompensating for a lack of movement in a plate, emphasizing the need for further investigation into potential

confounding factors. However, these findings would need to be further investigated in a plate-by-plate basis and validated through an independent experiment.

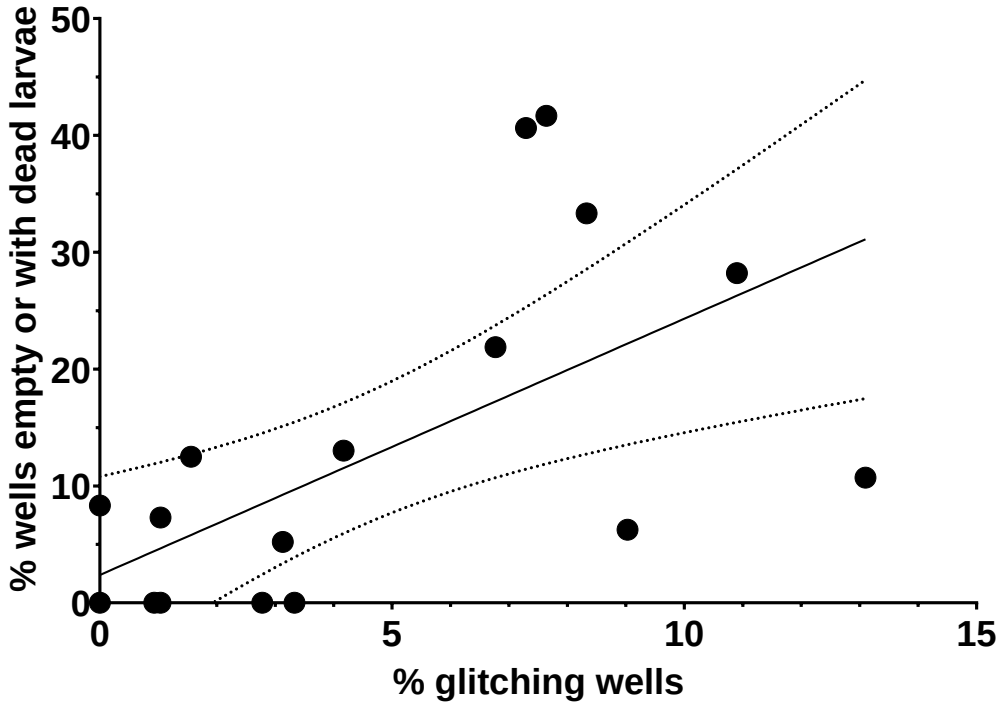

Figure B.1 – Correlation between the percentage of glitches occurring in behavioural testing and the percentage of wells empty or with dead larvae. Data in Table B.1 was analysed using the Pearson correlation coefficient (r = 0.6169, p = 0.0049) and simple regression analysis (slope = 2.193, p = 0.0049). Dots represent XY pairs, solid line the line of best fit and dotted lines the 95% Confidence Intervals.

Appendix C: Plate-by-plate comparison of the effect of alternating light-dark on zebrafish behaviour after exposure to CuSO4

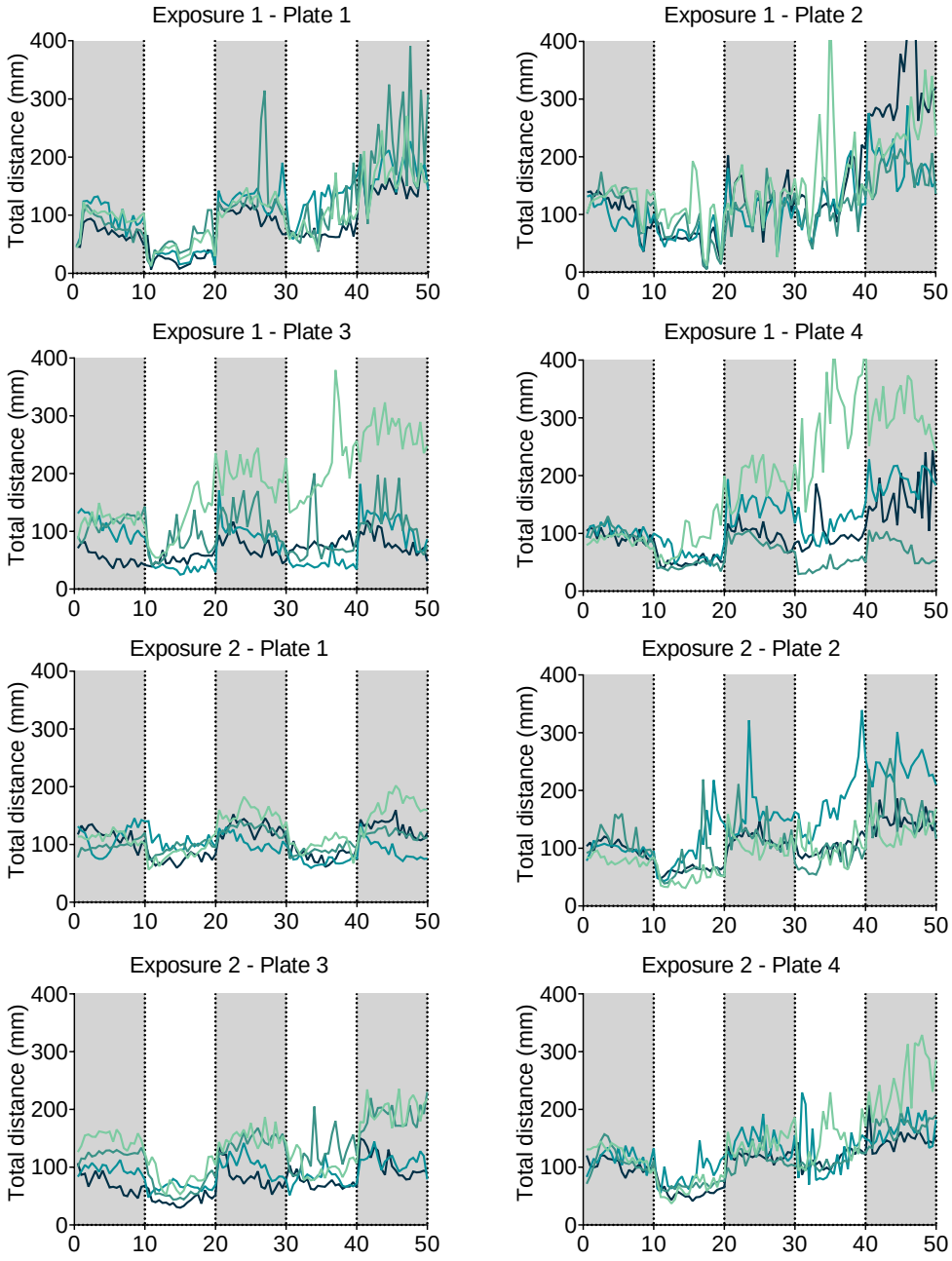

Figure C.1 – Plate by plate comparison of the effect of alternating light-dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 48-hour exposure to CuSO4. While CuSO4 was found to have a minor impact on phototaxis in all exposure groups, the study also revealed an unexpected lack of response to light stimuli in some larvae groups, including the control group, from the 30-minute marker. This phenomenon was observed in all plates of the first independent experiment but was absent in the second set of plates recorded.

Appendix D: Plate-by-plate comparison of the effect of alternating light-dark on zebrafish behaviour after co-exposure with DSS and DSP.

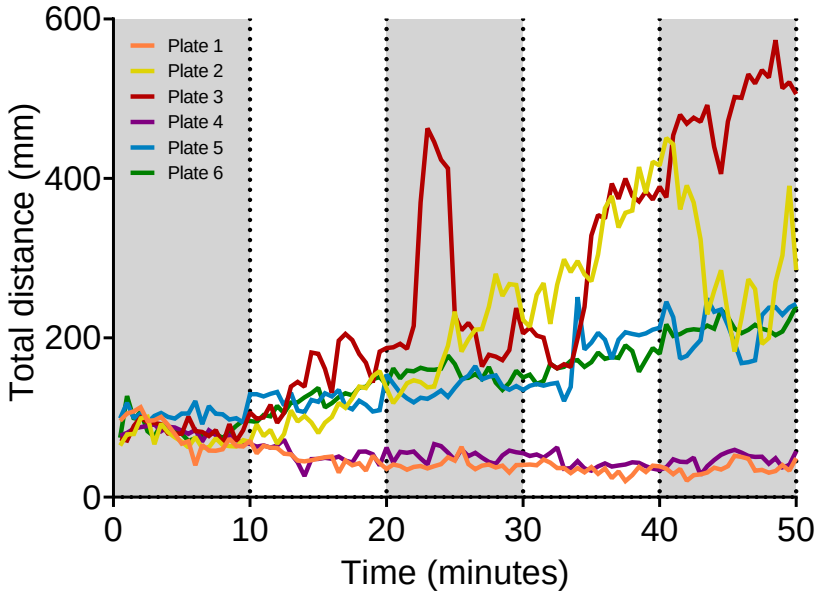

Figure D.1 – Plate by plate comparison of the effect of alternating light-dark periods on locomotion in Tg(mpx:GFP)i114 larval zebrafish after 10-day co-exposure with 0.33 mg/mL dextran sodium sulfate (DSS) and 132 µg/L DSP. Free swimming hatched larvae were introduced to the dilutions from 3dpf. An initial 10-minute acclimation period of darkness was followed by two alternating cycles of 10 minutes light and 10 minutes dark. Grey areas signify dark conditions. Data are presented as mean distance moved (in mm) in 30-second intervals throughout a 50-minute session. N=3 to 4 larvae per group.

Appendix E: Gene analytics pathway enrichment for CTD drug-gene interactions of SSRI and TCA antidepressants.

Table E.1 – Gene analytics pathway enrichment for comparative Toxicogenomics Database (CTD) drug-gene interactions of five SSRI antidepressants. Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. Yes denotes an interaction between the drug and the pathway was identified by this method, while no means no link was flagged. SRT = sertraline; PRX = paroxetine; FLX = fluoxetine; FLV = fluvoxamine; CIT = citalopram; SSRI = Selective serotonin reuptake inhibitors.

Pathway	Freq.	CIT	SRT	FLX	PRX	FLX
ERK Pathway in Huntingtons Disease	5	Yes	Yes	Yes	Yes	Yes
Gefitinib Pathway, Pharmacokinetics	5	Yes	Yes	Yes	Yes	Yes
Glucose / Energy Metabolism	5	Yes	Yes	Yes	Yes	Yes
Paroxetine Pathway, Pharmacokinetics	5	Yes	Yes	Yes	Yes	Yes
Phenytoin Pathway, Pharmacokinetics	5	Yes	Yes	Yes	Yes	Yes
Serotonergic Synapse	5	Yes	Yes	Yes	Yes	Yes
Statin Pathway - Generalized, Pharmacokinetics	5	Yes	Yes	Yes	Yes	Yes
Sudden Infant Death Syndrome Susceptibility Pathways	5	Yes	Yes	Yes	Yes	Yes
AGE-RAGE signalling Pathway in Diabetic Complications	4	No	Yes	Yes	Yes	Yes
AGE/RAGE Pathway	4	No	Yes	Yes	Yes	Yes
Allograft Rejection	4	No	Yes	Yes	Yes	Yes
Androstenedione and Testosterone Biosynthesis (...)	4	Yes	Yes	Yes	No	Yes
Brain-Derived Neurotrophic Factor (BDNF) signalling Pathway	4	Yes	Yes	Yes	Yes	No
CAMP signalling Pathway	4	Yes	Yes	Yes	Yes	No
Circadian Entrainment	4	Yes	Yes	Yes	Yes	No
Codeine and Morphine Pathway, Pharmacokinetics	4	Yes	Yes	No	Yes	Yes
Corticotropin-releasing Hormone signalling Pathway	4	Yes	Yes	Yes	Yes	No
Development VEGF signalling Via VEGFR2 - Generic Cascades	4	No	Yes	Yes	Yes	Yes
Gemtuzumab Ozogamicin Pathway, Pharmacokinetics/Pharmacodynamics	4	Yes	Yes	Yes	Yes	No
Hepatitis C and Hepatocellular Carcinoma	4	No	Yes	Yes	Yes	Yes
LT-BetaR Pathway	4	No	Yes	Yes	Yes	Yes
Monoamine Transport	4	Yes	Yes	Yes	Yes	No
Pertussis	4	No	Yes	Yes	Yes	Yes
Photodynamic Therapy-induced NF-kB Survival signalling	4	No	Yes	Yes	Yes	Yes
Selective Serotonin Reuptake Inhibitor Pathway, Phar. (...)	4	Yes	Yes	Yes	Yes	No

Table E.1 – Gene analytics pathway enrichment for CTD drug-gene interactions of five SSRI antidepressants. Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. (*Continued*)

Pathway	Freq.	CIT	SRT	FLX	PRX	FLX
Spinal Cord Injury	4	No	Yes	Yes	Yes	Yes
Tamoxifen Pathway, Pharmacokinetics	4	Yes	Yes	Yes	No	Yes
TNF signalling Pathway	4	No	Yes	Yes	Yes	Yes
Toxoplasmosis	4	No	Yes	Yes	Yes	Yes
Transmission Across Chemical Synapses	4	Yes	Yes	Yes	Yes	No
Tryptophan Utilization	4	Yes	Yes	Yes	No	Yes
VEGF Pathway (Tocris)	4	No	Yes	Yes	Yes	Yes
4-1BB Pathway	3	No	Yes	Yes	Yes	No
Activated TLR4 Signalling	3	No	Yes	Yes	Yes	No
Activation of BH3-only Proteins	3	No	Yes	Yes	Yes	No
Activation of Caspases Through Apoptosome-med. (...)	3	No	Yes	Yes	No	Yes
Adipogenesis	3	Yes	Yes	Yes	No	No
Agrin Interactions at Neuromuscular Junction	3	No	Yes	Yes	Yes	No
Akt signalling	3	No	Yes	Yes	Yes	No
Amphetamine Addiction	3	Yes	Yes	Yes	No	No
Amyotrophic Lateral Sclerosis (ALS)	3	Yes	Yes	Yes	No	No
Angiopoietin Like Protein 8 Regulatory Pathway	3	No	Yes	Yes	Yes	No
Apoptosis and Survival Caspase Cascade	3	No	Yes	Yes	Yes	No
Apoptosis and Survival_Anti-apoptotic Action of Nuc. (...)	3	No	Yes	Yes	Yes	No
Apoptosis Modulation and signalling	3	No	Yes	Yes	Yes	No
Apoptosis Pathway	3	No	Yes	Yes	Yes	No
Apoptotic Pathways in Synovial Fibroblasts	3	No	Yes	Yes	Yes	No
Aryl Hydrocarbon Receptor	3	No	Yes	Yes	Yes	No
ATF-2 Transcription Factor Network	3	No	Yes	Yes	Yes	No
BDNF-TrkB signalling	3	No	Yes	Yes	Yes	No
Beta-Adrenergic signalling	3	No	Yes	Yes	Yes	No
CCR5 Pathway in Macrophages	3	No	Yes	Yes	Yes	No
Celecoxib Pathway, Pharmacodynamics	3	No	Yes	Yes	Yes	No
Ceramide Pathway	3	No	Yes	Yes	Yes	No
Ceramide signalling Pathway	3	No	Yes	Yes	Yes	No
Colorectal Cancer Metastasis	3	No	Yes	Yes	Yes	No
Common Cytokine Receptor Gamma-Chain Family (...)	3	No	Yes	Yes	Yes	No
CREB Pathway	3	No	Yes	Yes	Yes	No

Table E.1 – Gene analytics pathway enrichment for CTD drug-gene interactions of five SSRI antidepressants. Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. (*Continued*)

Pathway	Freq.	CIT	SRT	FLX	PRX	FLX
CXCR3-mediated signalling Events	3	No	Yes	Yes	Yes	No
Cytochrome P450 - Arranged By Substrate Type	3	Yes	Yes	No	No	Yes
Cytokine signalling in Immune System	3	No	Yes	Yes	Yes	No
Development A3 Receptor signalling	3	No	Yes	Yes	Yes	No
Development Dopamine D2 Receptor Transactivation (...)	3	No	Yes	Yes	Yes	No
Development EGFR signalling Via Small GTPases	3	No	Yes	Yes	Yes	No
Development Endothelin-1/EDNRA signalling	3	No	Yes	Yes	Yes	No
Development EPO-induced Jak-STAT Pathway	3	No	Yes	Yes	Yes	No
Development ERBB-family signalling	3	No	Yes	Yes	Yes	No
Development HGF signalling Pathway	3	No	Yes	Yes	Yes	No
Development IGF-1 Receptor signalling	3	No	Yes	Yes	Yes	No
Development Ligand-independent Activation of ESR1 (...)	3	No	Yes	Yes	Yes	No
Development_Leptin signalling Via JAK/STAT and (...)	3	No	Yes	Yes	Yes	No
Dimerization of Procaspase-8	3	No	Yes	Yes	Yes	No
Downstream signalling Events of B Cell Receptor (BCR)	3	No	Yes	Yes	Yes	No
Downstream signalling in Naive CD8+ T Cells	3	No	Yes	Yes	Yes	No
DREAM Repression and Dynorphin Expression	3	No	Yes	Yes	Yes	No
EBV LMP1 signalling	3	No	Yes	Yes	Yes	No
Endometrial Cancer	3	No	Yes	Yes	Yes	No
ErbB signalling Pathway	3	No	Yes	Yes	Yes	No
ErbB1 Downstream signalling	3	No	Yes	Yes	Yes	No
ERK signalling	3	No	Yes	Yes	Yes	No
FMLP Pathway	3	No	Yes	Yes	Yes	No
Focal Adhesion	3	No	Yes	Yes	Yes	No
Follicle Stimulating Hormone (FSH) signalling Pathway	3	No	Yes	Yes	Yes	No
FoxO signalling Pathway	3	No	Yes	Yes	Yes	No
G-Beta Gamma signalling	3	No	Yes	Yes	Yes	No
G-protein signalling RAC1 in Cellular Process	3	No	Yes	Yes	Yes	No
G-protein signalling Ras Family GTPases in Kinase (...)	3	No	Yes	Yes	Yes	No
GAB1 Signalosome	3	No	Yes	Yes	Yes	No
GABAergic Synapse	3	No	Yes	Yes	Yes	No
GDNF-Family Ligands and Receptor Interactions	3	No	Yes	Yes	Yes	No
Glioma	3	No	Yes	Yes	Yes	No

Table E.1 – Gene analytics pathway enrichment for CTD drug-gene interactions of five SSRI antidepressants. Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. (*Continued*)

Pathway	Freq.	CIT	SRT	FLX	PRX	FLX
Glucocorticoid Receptor Regulatory Network	3	Yes	Yes	Yes	No	No
GPCR Pathway	3	No	Yes	Yes	Yes	No
Hepatitis C	3	No	Yes	Yes	Yes	No
HIF-1 signalling Pathway	3	No	Yes	Yes	Yes	No
IL-17 Family signalling Pathways	3	No	Yes	Yes	Yes	No
IL-2 Gene Expression in Activated and Quiescent T-Cells	3	No	Yes	Yes	Yes	No
IL-2 Pathway	3	No	Yes	Yes	Yes	No
IL-2 signalling Pathway	3	No	Yes	Yes	Yes	No
IL-7 signalling Pathway	3	No	Yes	Yes	Yes	No
Immune Response Fc Epsilon RI Pathway	3	No	Yes	Yes	Yes	No
Immune Response IL-23 signalling Pathway	3	No	Yes	Yes	Yes	No
Immune Response_IL-6 signalling Pathway	3	No	Yes	Yes	Yes	No
Influenza A	3	No	Yes	Yes	Yes	No
Innate Immune System	3	No	Yes	Yes	Yes	No
Integrated Breast Cancer Pathway	3	No	Yes	Yes	Yes	No
Integrin Pathway	3	No	Yes	Yes	Yes	No
Integrins in Angiogenesis	3	No	Yes	Yes	Yes	No
Interleukin-4 and 13 signalling	3	No	Yes	Yes	No	Yes
Kit Receptor signalling Pathway	3	No	Yes	Yes	Yes	No
Legionellosis	3	No	Yes	Yes	Yes	No
Linoleic Acid Metabolism	3	No	Yes	Yes	No	Yes
MAPK Pathway	3	No	Yes	Yes	Yes	No
MAPK signalling Pathway	3	No	Yes	Yes	Yes	No
MAPK signalling: Mitogen Stimulation Pathway	3	No	Yes	Yes	Yes	No
Melanocyte Development and Pigmentation	3	No	Yes	Yes	Yes	No
Metabolism	3	Yes	No	Yes	No	Yes
MicroRNAs in Cancer	3	No	Yes	Yes	Yes	No
MicroRNAs in Cardiomyocyte Hypertrophy	3	No	Yes	Yes	Yes	No
Nanog in Mammalian ESC Pluripotency	3	No	Yes	Yes	Yes	No
Nanomaterial Induced Apoptosis	3	No	Yes	Yes	Yes	No
Neuroscience	3	Yes	No	Yes	Yes	No
NF-kappa B signalling Pathway	3	No	Yes	Yes	No	Yes
NFAT and Cardiac Hypertrophy	3	No	Yes	Yes	Yes	No

Table E.1 – Gene analytics pathway enrichment for CTD drug-gene interactions of five SSRI antidepressants. Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. (*Continued*)

Pathway	Freq.	CIT	SRT	FLX	PRX	FLX
NGF Pathway	3	No	Yes	Yes	Yes	No
Nuclear Events (kinase and Transcription Factor (...)	3	No	Yes	Yes	Yes	No
Oocyte Meiosis	3	No	Yes	Yes	Yes	No
Osteopontin-mediated Events	3	No	Yes	Yes	Yes	No
P53 signalling	3	No	Yes	Yes	Yes	No
P70S6K signalling	3	No	Yes	Yes	Yes	No
P75(NTR)-mediated signalling	3	No	Yes	Yes	Yes	No
PAK Pathway	3	No	Yes	Yes	Yes	No
Pancreatic Cancer	3	No	Yes	Yes	Yes	No
Pathways in Cancer	3	No	Yes	Yes	Yes	No
PEDF Induced signalling	3	No	Yes	Yes	Yes	No
Peptide Ligand-binding Receptors	3	Yes	No	Yes	Yes	No
Phospholipase D signalling Pathway	3	No	Yes	Yes	Yes	No
Physiological and Pathological Hypertrophy of The Heart	3	No	Yes	Yes	Yes	No
PI3K-Akt signalling Pathway	3	No	Yes	Yes	Yes	No
Platinum Drug Resistance	3	No	Yes	Yes	Yes	No
Prolactin signalling Pathway	3	No	Yes	Yes	Yes	No
Proteoglycans in Cancer	3	No	Yes	Yes	Yes	No
RANK signalling in Osteoclasts	3	No	Yes	Yes	Yes	No
Regulation of Lipid Metabolism Insulin signalling-gen. (...)	3	No	Yes	Yes	Yes	No
Respiratory Electron Transport, ATP Synthesis (...)	3	No	Yes	Yes	Yes	No
RET signalling	3	No	Yes	Yes	Yes	No
Retinoic Acid Receptors-mediated signalling	3	No	Yes	Yes	Yes	No
S-1P Stimulated signalling	3	No	Yes	Yes	Yes	No
S1P3 Pathway	3	No	Yes	Yes	Yes	No
Serotonin Receptor 2 and ELK-SRF/GATA4 signalling	3	No	Yes	Yes	Yes	No
signalling By GPCR	3	No	Yes	Yes	Yes	No
signalling Events Regulated By Ret Tyrosine Kinase	3	No	Yes	Yes	Yes	No
signalling in Gap Junctions	3	No	Yes	Yes	Yes	No
TGF-Beta Pathway	3	No	Yes	Yes	Yes	No
TGF-beta signalling Pathways	3	No	Yes	Yes	Yes	No
Th17 Cell Differentiation	3	No	Yes	Yes	Yes	No
TNF signalling (sino)	3	No	Yes	Yes	Yes	No

Table E.1 – Gene analytics pathway enrichment for CTD drug-gene interactions of five SSRI antidepressants. Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. (*Continued*)

Pathway	Freq.	CIT	SRT	FLX	PRX	FLX
TNFR1 Pathway	3	No	Yes	Yes	Yes	No
Toll Comparative Pathway	3	No	Yes	Yes	Yes	No
Toll-like Receptor signalling Pathway	3	No	Yes	Yes	Yes	No
Toll-Like Receptor signalling Pathways	3	No	Yes	Yes	Yes	No
TRAF Pathway	3	No	Yes	Yes	Yes	No
Transcription Androgen Receptor Nuclear signalling	3	No	Yes	Yes	Yes	No
Transcription Factor Regulation in Adipogenesis	3	No	Yes	Yes	Yes	No
Transcription_Role of VDR in Regulation of Genes (...)	3	No	Yes	Yes	No	Yes
Tuberculosis	3	No	Yes	Yes	Yes	No
Type II Diabetes Mellitus	3	No	Yes	Yes	Yes	No
UVA-Induced MAPK signalling	3	No	Yes	Yes	Yes	No
Vascular Smooth Muscle Contraction	3	No	Yes	Yes	Yes	No
VEGF Pathway (Qiagen)	3	No	Yes	Yes	Yes	No
Viral Carcinogenesis	3	No	Yes	Yes	Yes	No

Table E.2 – Gene analytics pathway enrichment for CTD drug-gene interactions of five tricyclic antidepressants (TCAs). Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. AMI = amitriptyline; NRT = nortriptylene; CLM = clomipramine; IMI = imipramine; DXP = doxepin; TCA = Tricyclic antidepressants.

Pathway	Freq.	AMI	NRT	CLO	IMI	DXP
Etoposide Pathway, Pharmacokinetics/Pharmacod. (...)	5	Yes	Yes	Yes	Yes	Yes
Acetaminophen Pathway (therapeutic Doses), Phar. (...)	4	Yes	Yes	Yes	Yes	No
Allograft Rejection	4	Yes	Yes	Yes	Yes	No
Codeine and Morphine Pathway, Pharmacokinetics	4	Yes	Yes	Yes	Yes	No
Constitutive Androstane Receptor Pathway	4	Yes	Yes	Yes	Yes	No
Cytochrome P450 - Arranged By Substrate Type	4	Yes	Yes	Yes	Yes	No
Drug Metabolism - Cytochrome P450	4	Yes	Yes	Yes	Yes	No
Gefitinib Pathway, Pharmacokinetics	4	Yes	Yes	Yes	Yes	No
Glucose / Energy Metabolism	4	Yes	Yes	Yes	Yes	No
Irinotecan Pathway	4	Yes	Yes	Yes	Yes	No
Legionellosis	4	Yes	Yes	Yes	Yes	No
Metabolism	4	Yes	Yes	Yes	Yes	No
Paroxetine Pathway, Pharmacokinetics	4	Yes	Yes	Yes	Yes	No
Pertussis	4	Yes	Yes	Yes	Yes	No
Phenytoin Pathway, Pharmacokinetics	4	Yes	Yes	Yes	Yes	No
Statin Pathway - Generalized, Pharmacokinetics	4	Yes	Yes	Yes	Yes	No
Sudden Infant Death Syndrome Susceptibility Pathways	4	Yes	Yes	Yes	Yes	No
Tamoxifen Pathway, Pharmacokinetics	4	Yes	Yes	Yes	Yes	No
Abacavir Transport and Metabolism	3	No	Yes	No	Yes	Yes
Amino Acid Metabolism	3	Yes	No	Yes	Yes	No
Apoptosis and Survival Caspase Cascade	3	No	Yes	Yes	Yes	No
Bile Secretion	3	Yes	Yes	No	Yes	No
Ceramide Pathway	3	No	Yes	Yes	Yes	No
DREAM Repression and Dynorphin Expression	3	No	Yes	Yes	Yes	No
Gemtuzumab Ozogamicin Pathway, Pharmacoki. (...)	3	Yes	Yes	Yes	No	No
Granzyme Pathway	3	No	Yes	Yes	Yes	No
Immune Response IL-23 signalling Pathway	3	Yes	No	Yes	Yes	No
Linoleic Acid Metabolism	3	Yes	Yes	No	Yes	No
Naphthalene Metabolism	3	Yes	Yes	No	Yes	No
Neurotransmitter Clearance In The Synaptic Cleft	3	Yes	Yes	No	Yes	No
Nuclear Receptors in Lipid Metabolism and Toxicity	3	Yes	Yes	No	Yes	No
Serotonergic Synapse	3	Yes	Yes	No	Yes	No

Table E.2 – Gene analytics pathway enrichment for CTD drug-gene interactions of five TCAs. Due to the extensive list obtained, only pathways shared by at least 3 drugs were included. Yes denotes an interaction between the drug and the pathway was identified by this method, while no means no link was flagged. AMI = amitriptyline; NRT = nortriptylene; CLM = clomipramine; IMI = imipramine; DXP = doxepin; TCA = Tricyclic antidepressants. (*Continued*)

Pathway	Freq.	AMI	NRT	CLO	IMI	DXP
Spinal Cord Injury	3	Yes	Yes	No	Yes	No
SREBF and MiR33 in Cholesterol and Lipid Homeostasis	3	Yes	No	Yes	Yes	No
TNFR1 Pathway	3	No	Yes	Yes	Yes	No
Toxoplasmosis	3	Yes	Yes	No	Yes	No
Tryptophan Utilization	3	Yes	No	Yes	Yes	No

Appendix F: Functional enrichment analysis of the differential gene expression profile for 20 antidepressants using Reactome

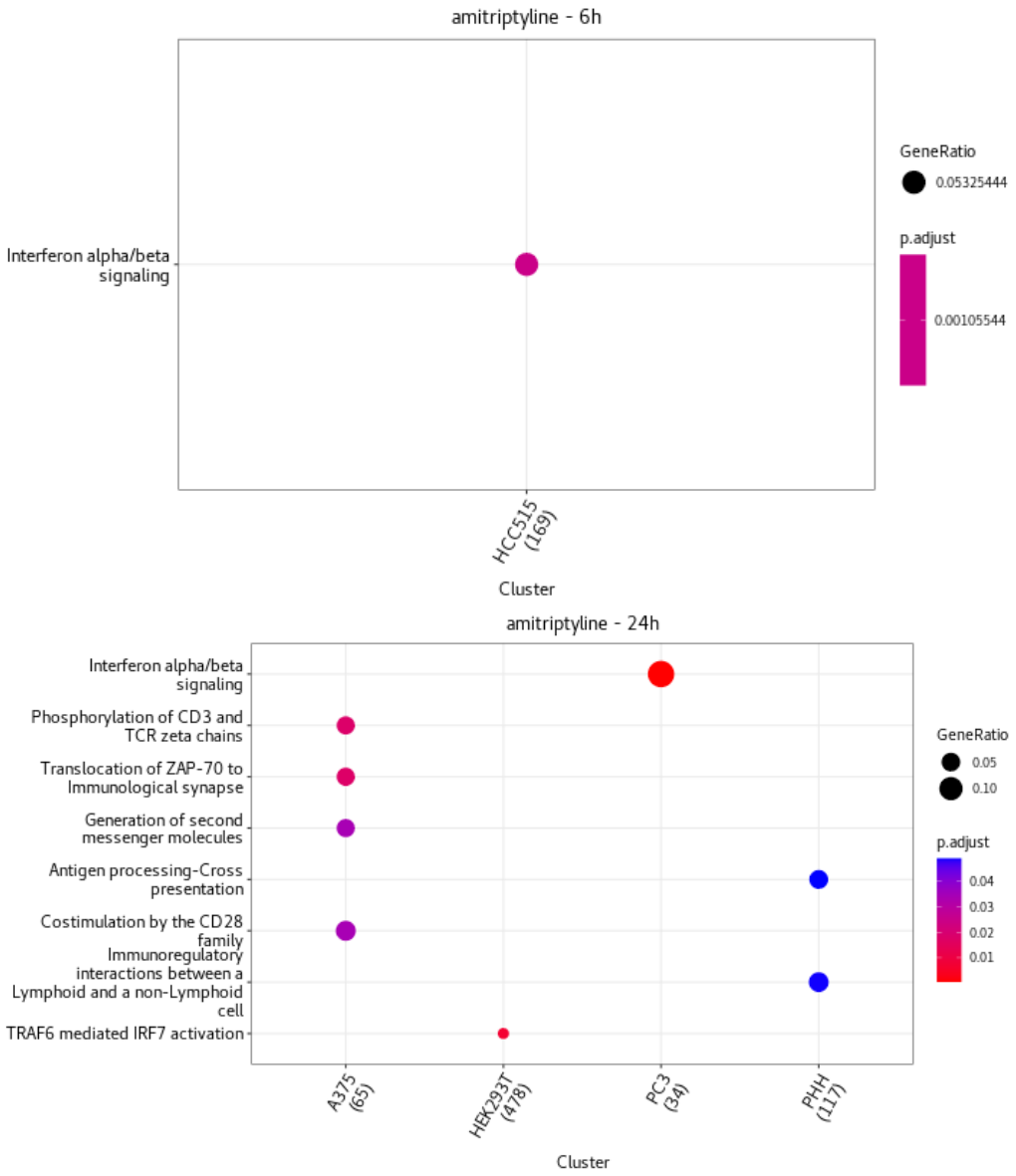

Figure F.1 – Functional enrichment analysis of the differential gene expression profile for antidepressant amitriptyline using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

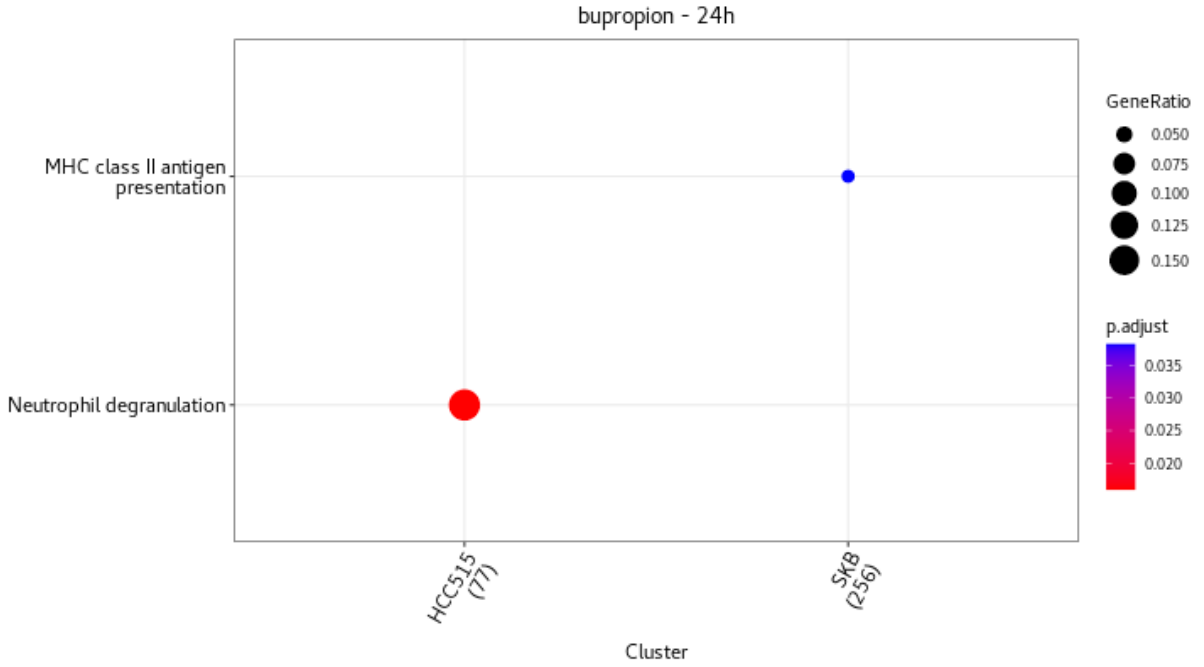

Figure F.2 – Functional enrichment analysis of the differential gene expression profile for antidepressant bupropion using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

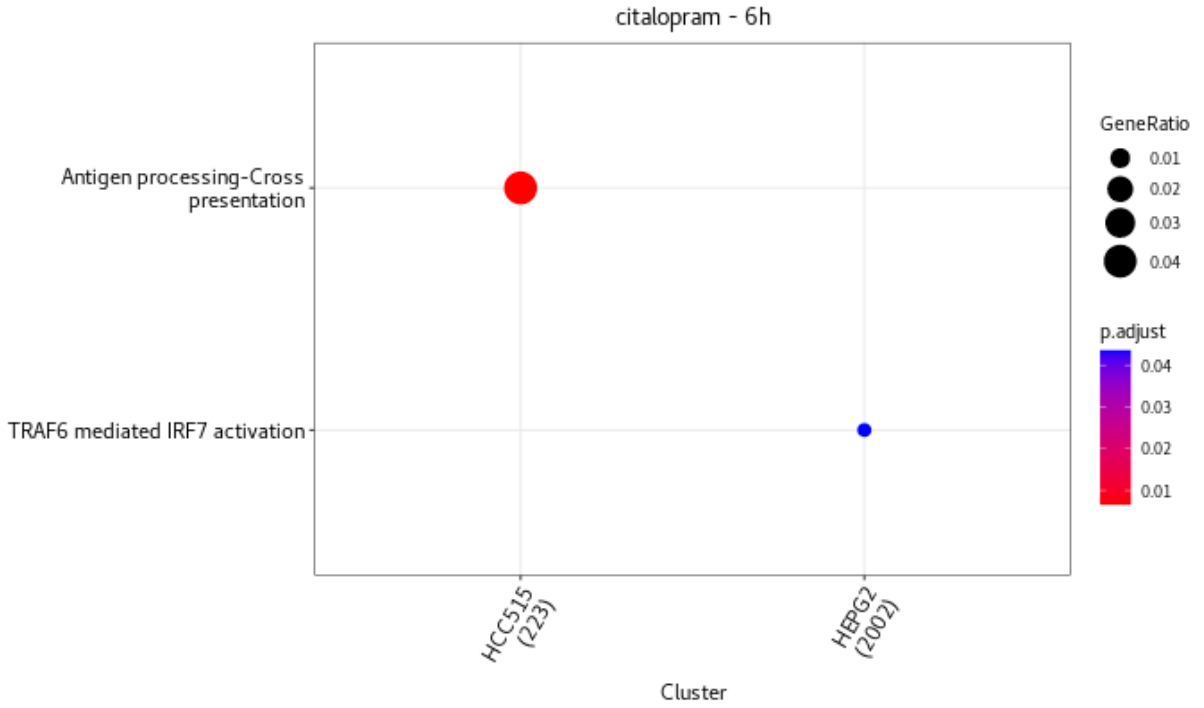

Figure F.3 – Functional enrichment analysis of the differential gene expression profile for antidepressant citalopram using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

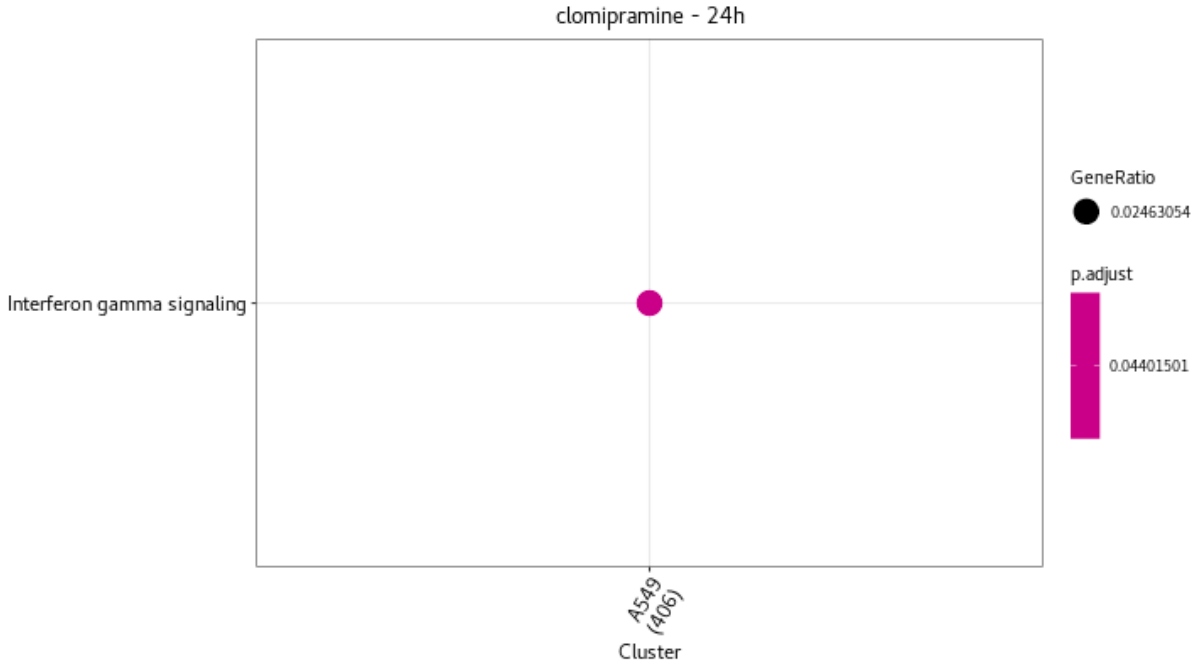

Figure F.4 – Functional enrichment analysis of the differential gene expression profile for antidepressant clomipramine using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

F.1 References

Fabregat, A., Jupe, S., Matthews, L., Sidiropoulos, K., Gillespie, M., Garapati, P., Haw, R., Jassal, B., Korninger, F., May, B., Milacic, M., Roca, C. D., Rothfels, K., Sevilla, C., Shamovsky, V., Shorser, S., Varusai, T., Viteri, G., Weiser, J., Wu, G., Stein, L., Hermjakob, H., and D'Eustachio, P. The Reactome Pathway Knowledgebase. *Nucleic Acids Research*, 46(D1):D649–D655, 1 2018. ISSN 0305-1048. doi: 10.1093/nar/gkx1132.

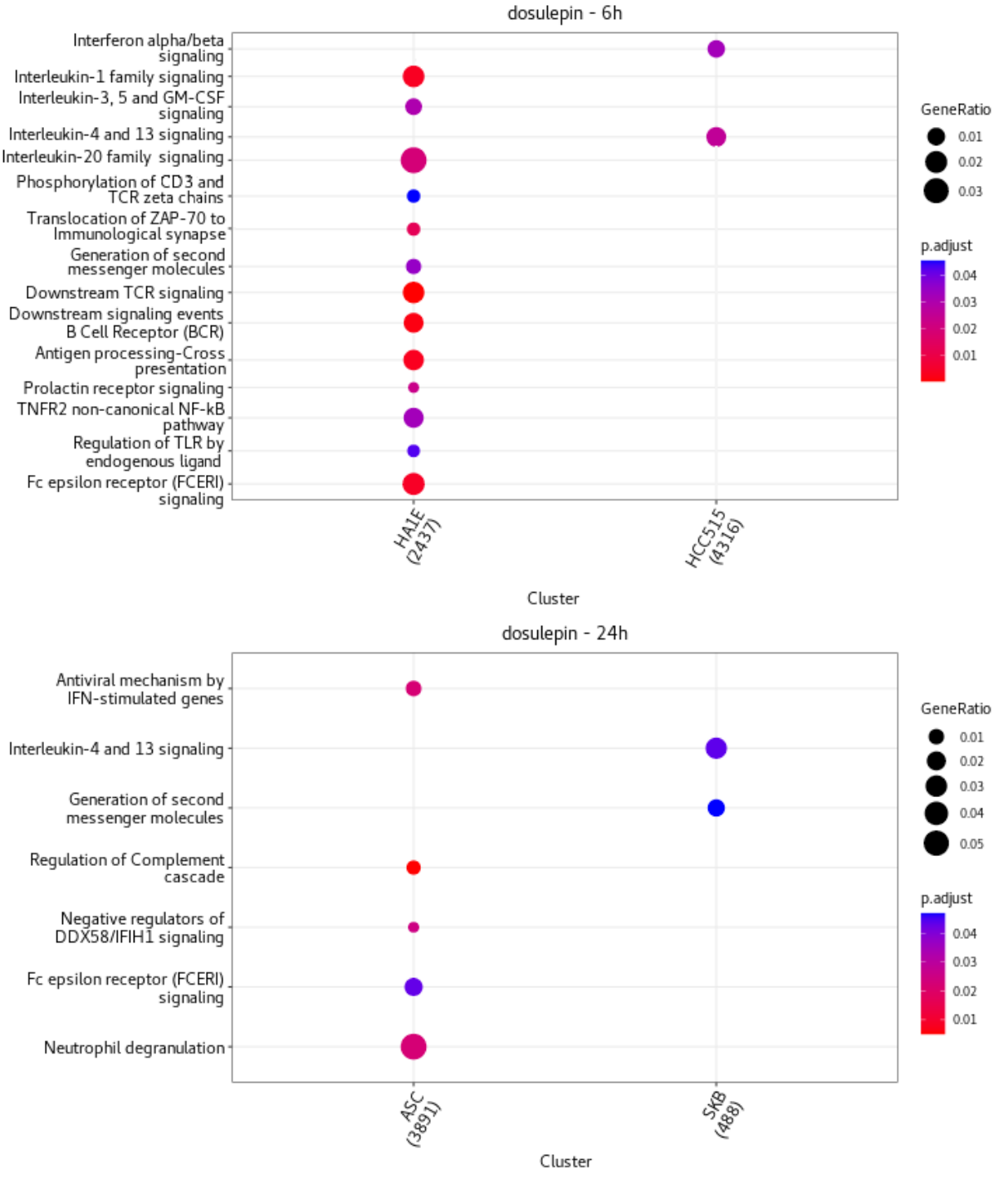

Figure F.5 – Functional enrichment analysis of the differential gene expression profile for antidepressant dosulepin using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

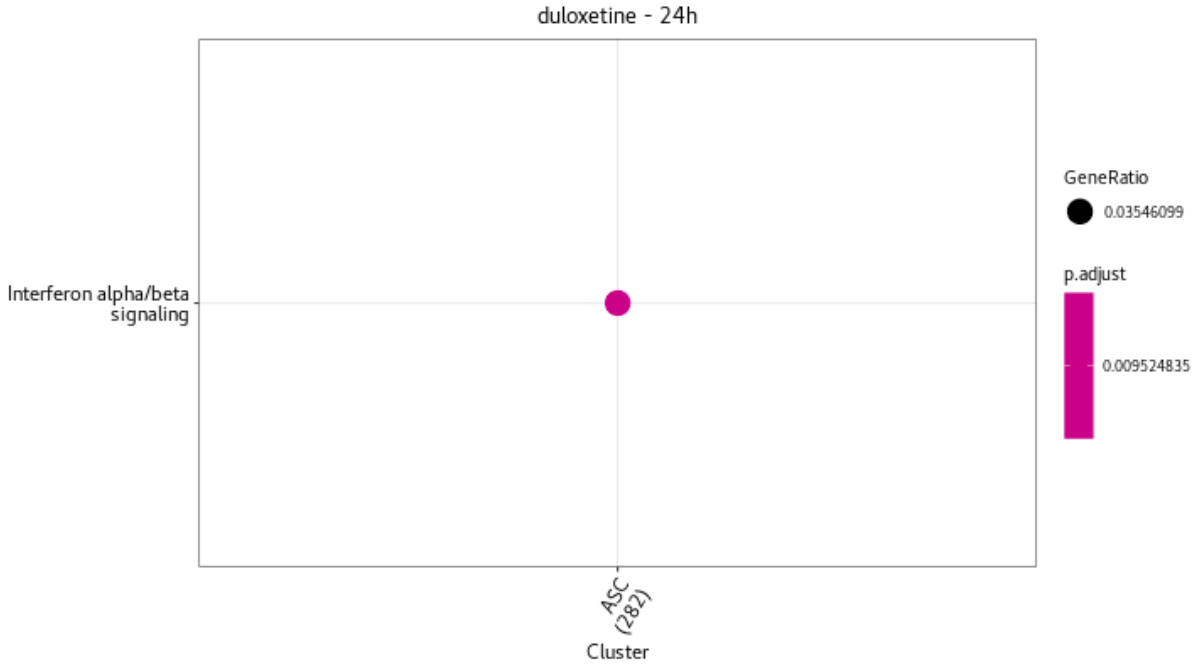

Figure F.6 – Functional enrichment analysis of the differential gene expression profile for antidepressant duloxetine using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

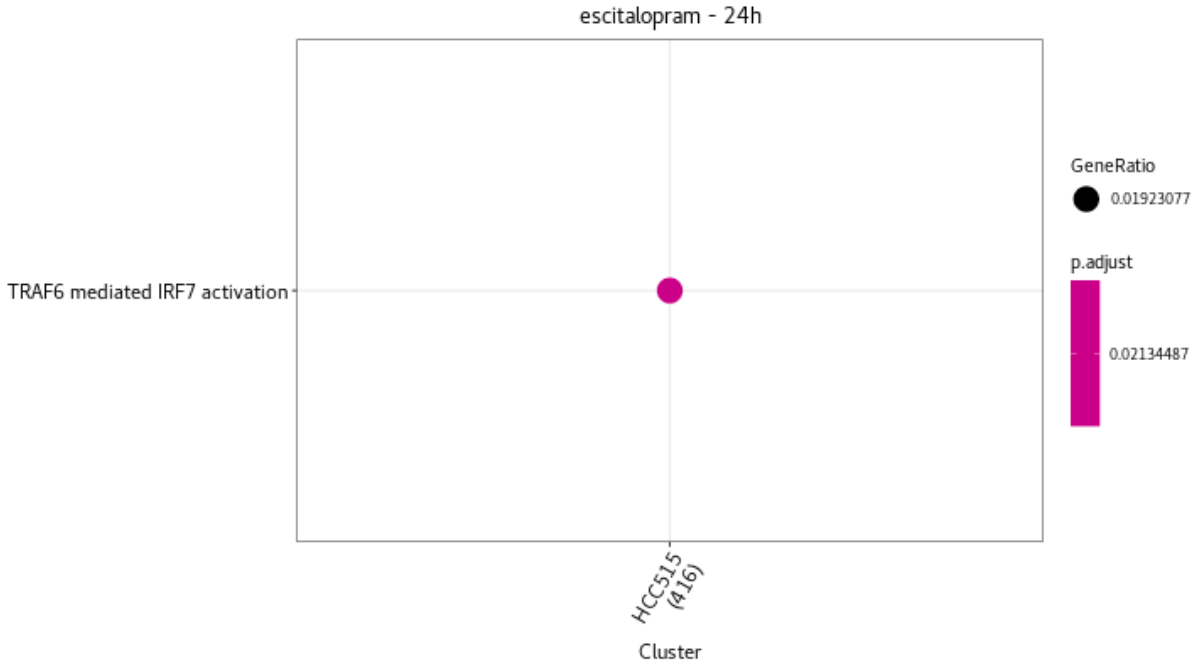

Figure F.7 – Functional enrichment analysis of the differential gene expression profile for antidepressant escitalopram using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

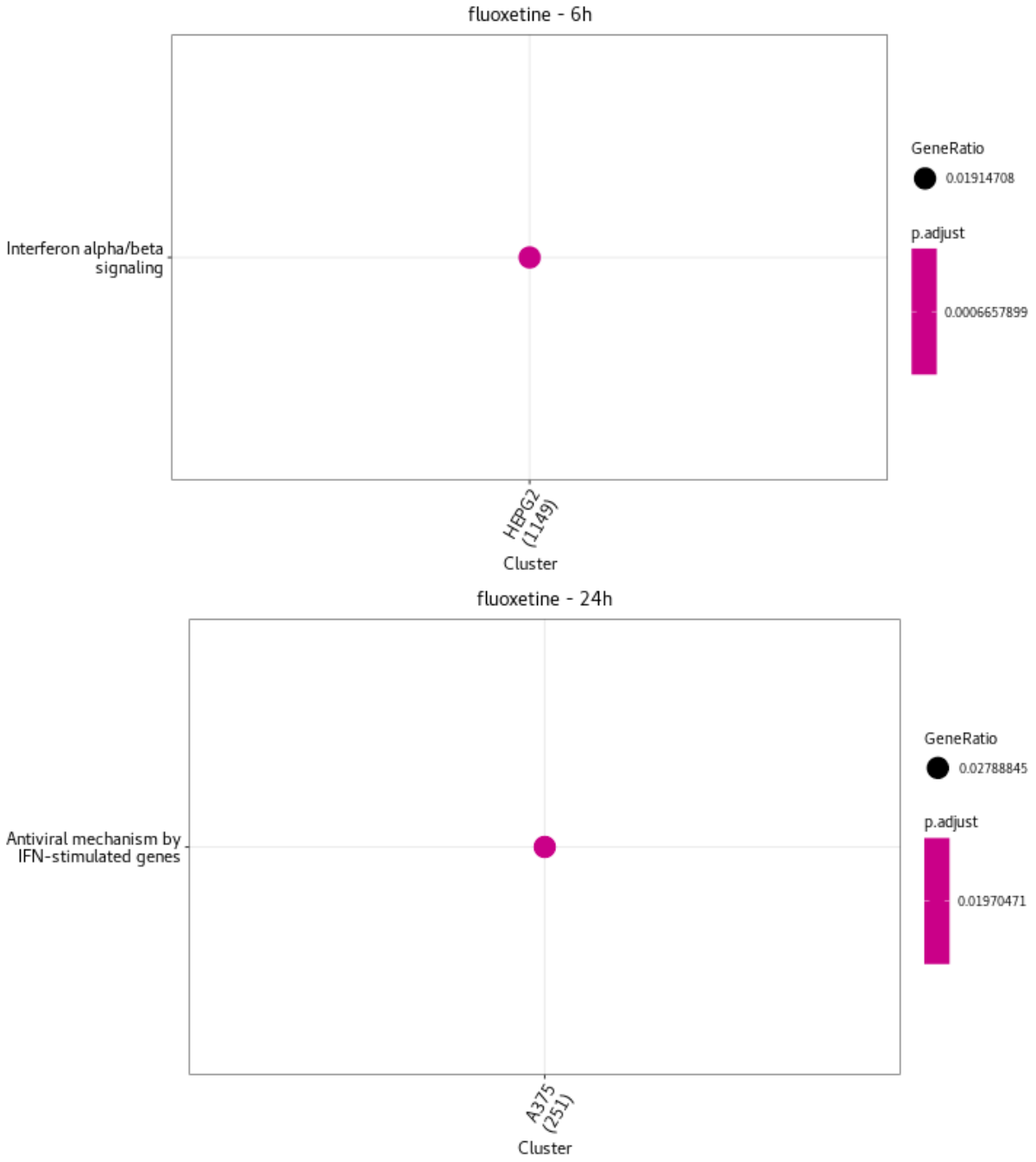

Figure F.8 – Functional enrichment analysis of the differential gene expression profile for antidepressant fluoxetine using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

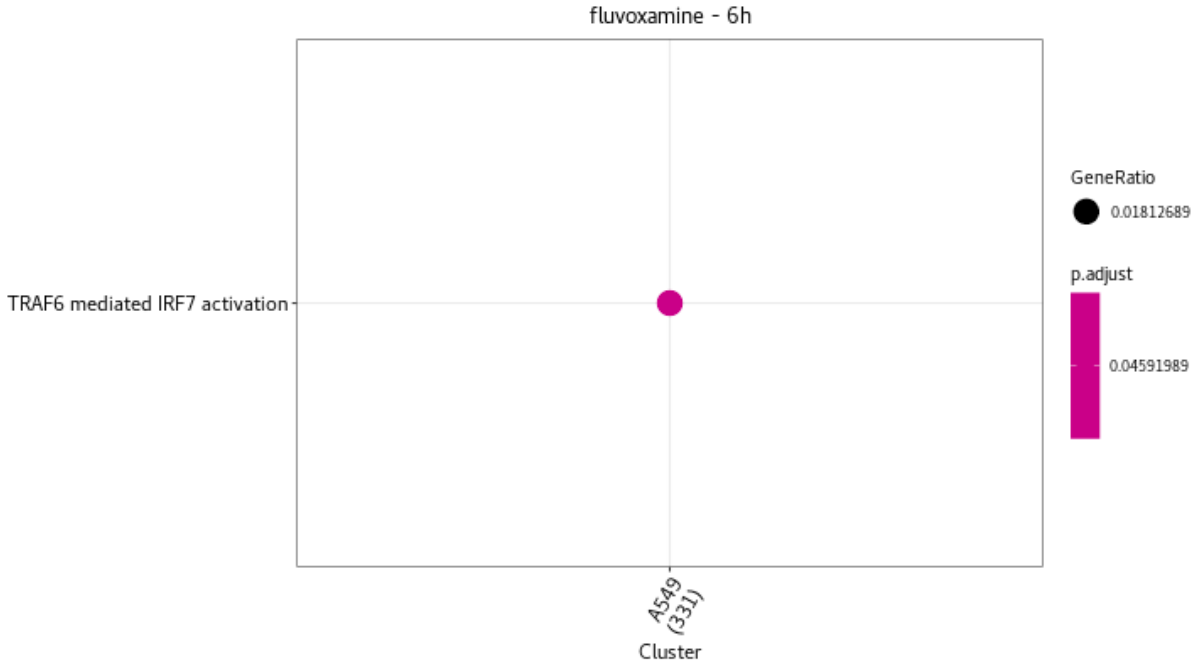

Figure F.9 – Functional enrichment analysis of the differential gene expression profile for antidepressant fluvoxamine using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

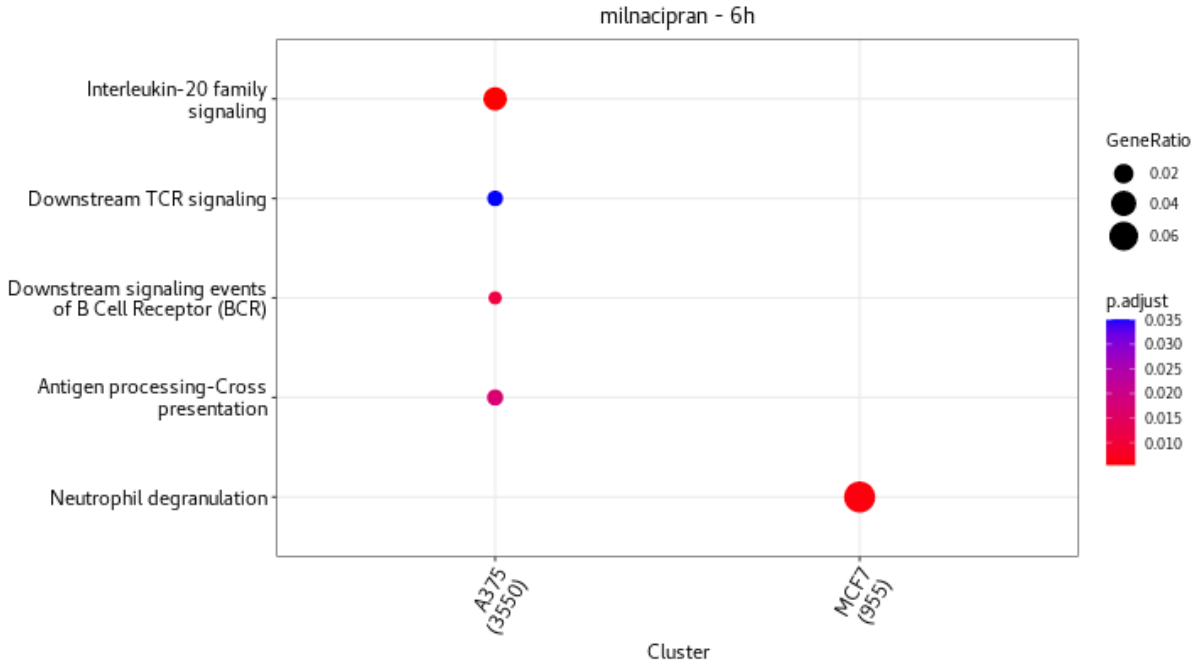

Figure F.10 – Functional enrichment analysis of the differential gene expression profile for antidepressant milnacipran using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

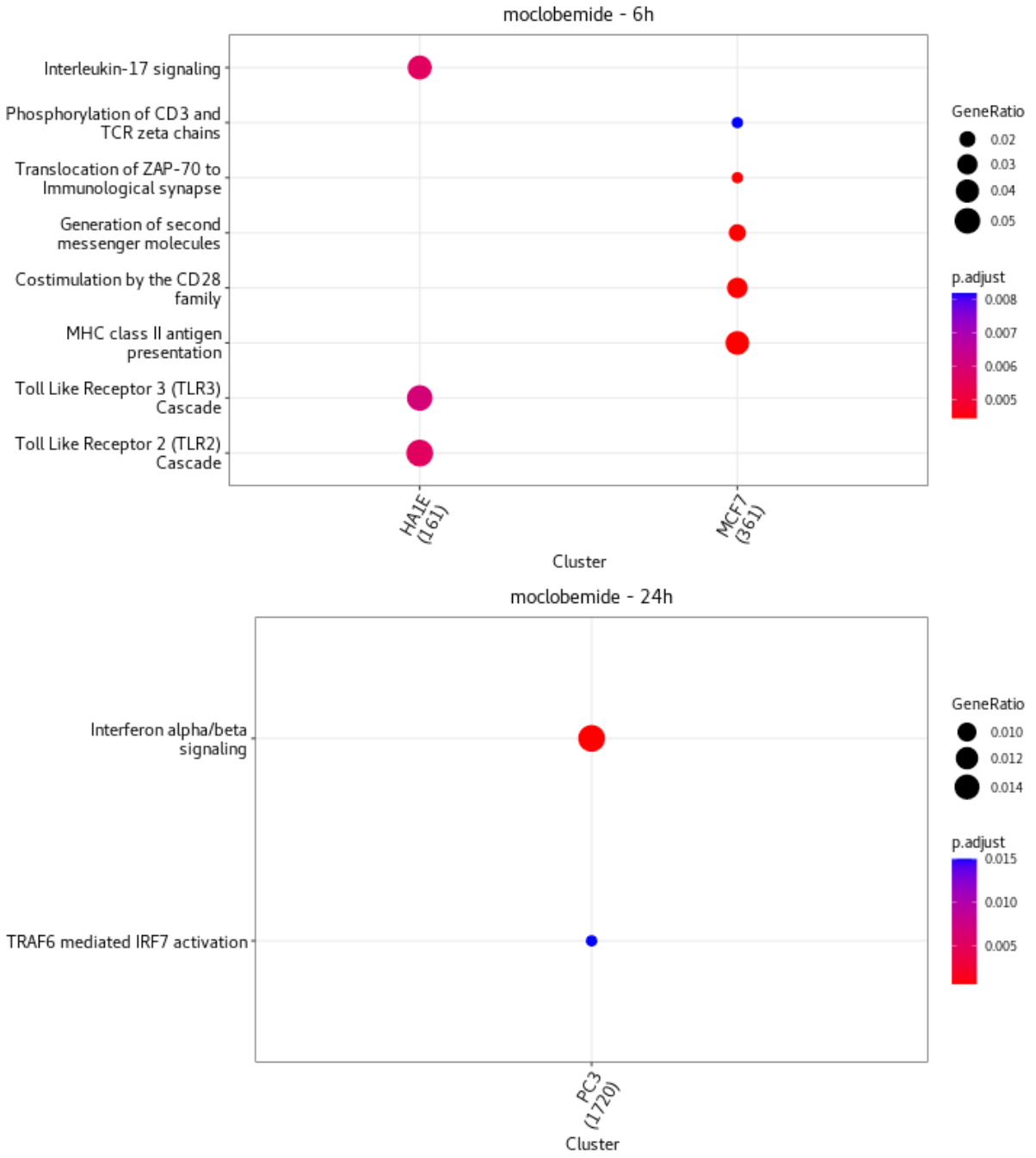

Figure F.11 – Functional enrichment analysis of the differential gene expression profile for antidepressant moclebamide using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

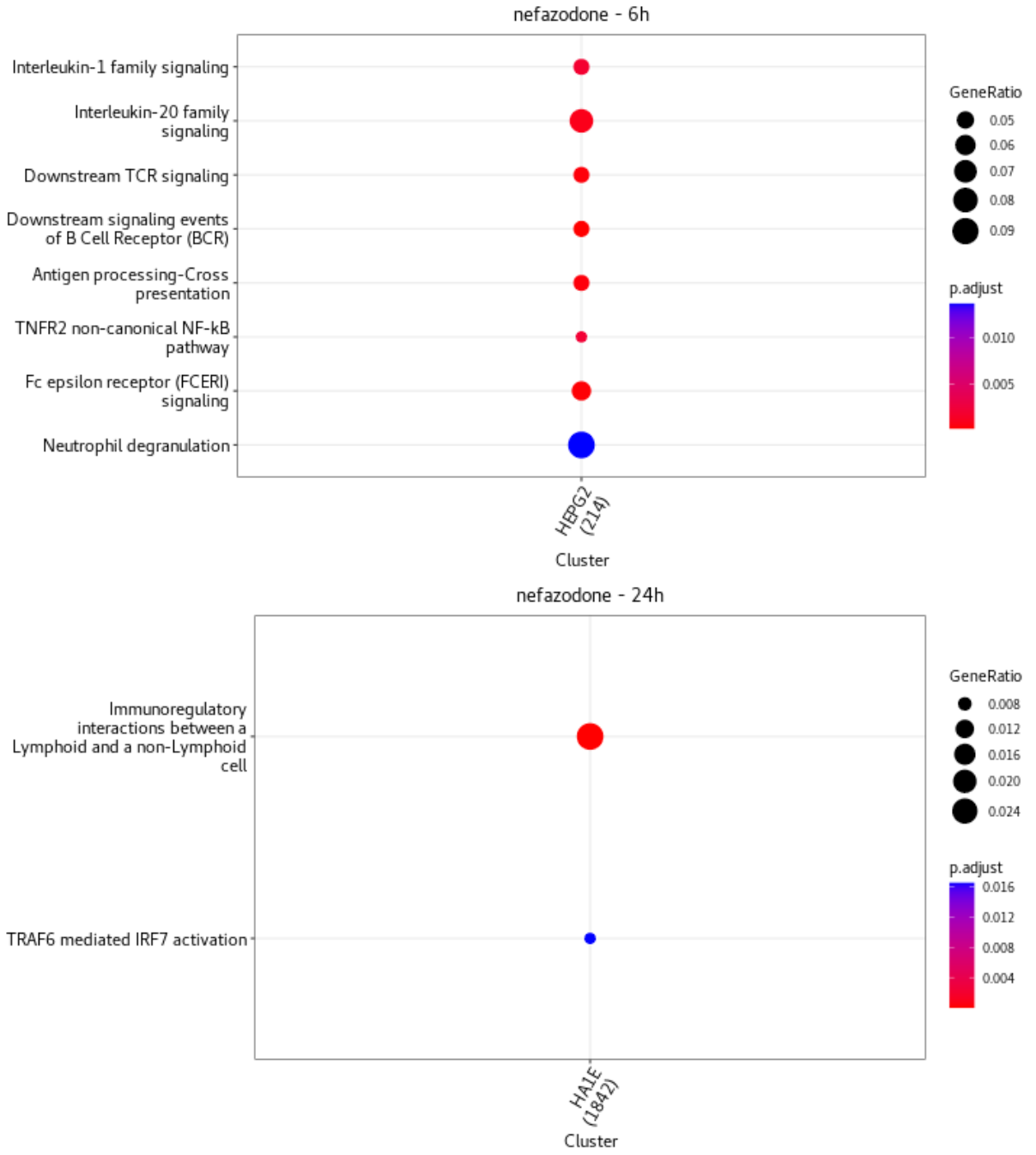

Figure F.12 – Functional enrichment analysis of the differential gene expression profile for antidepressant nefazodone using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

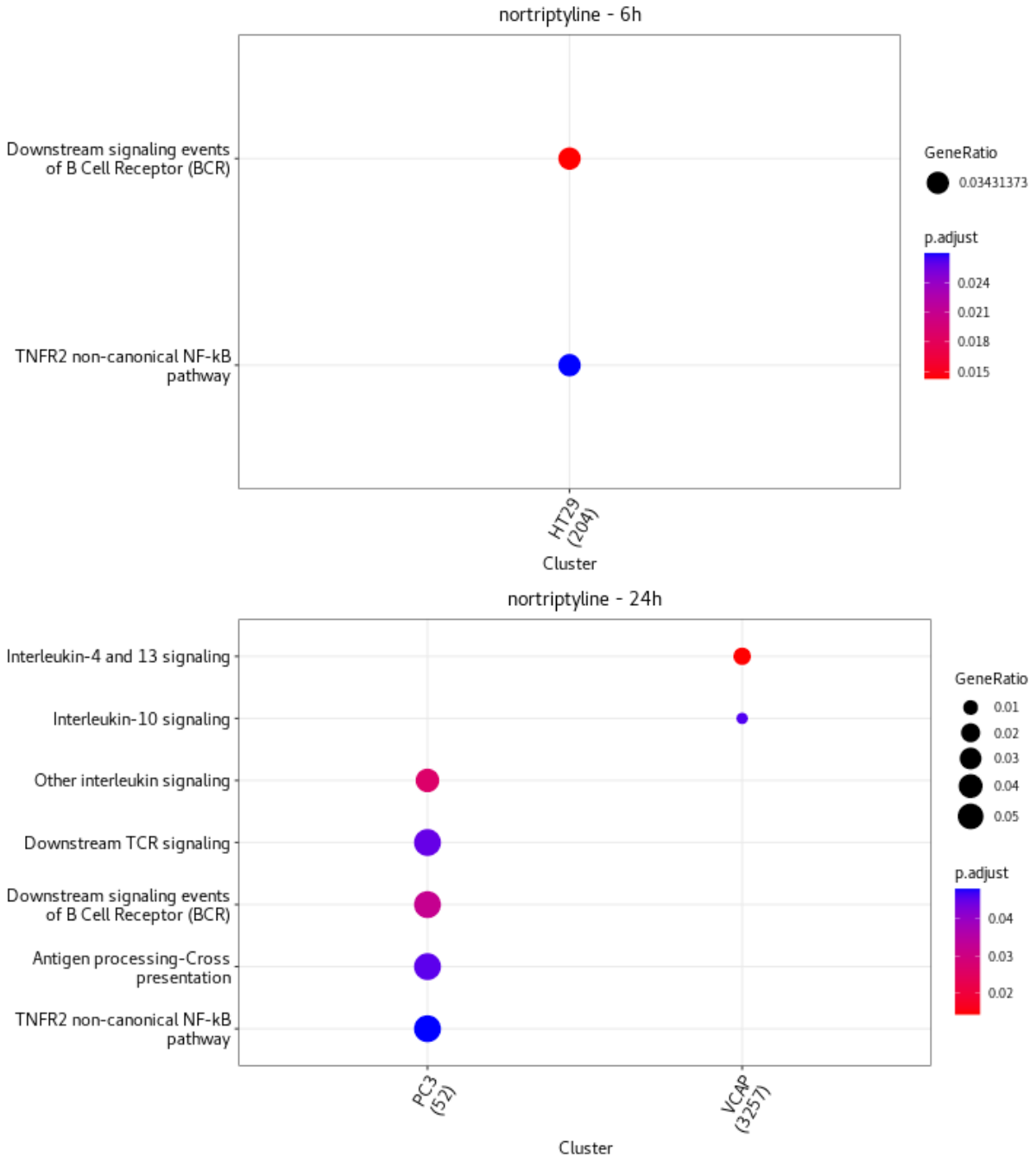

Figure F.13 – Functional enrichment analysis of the differential gene expression profile for antidepressant nortriptyline using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

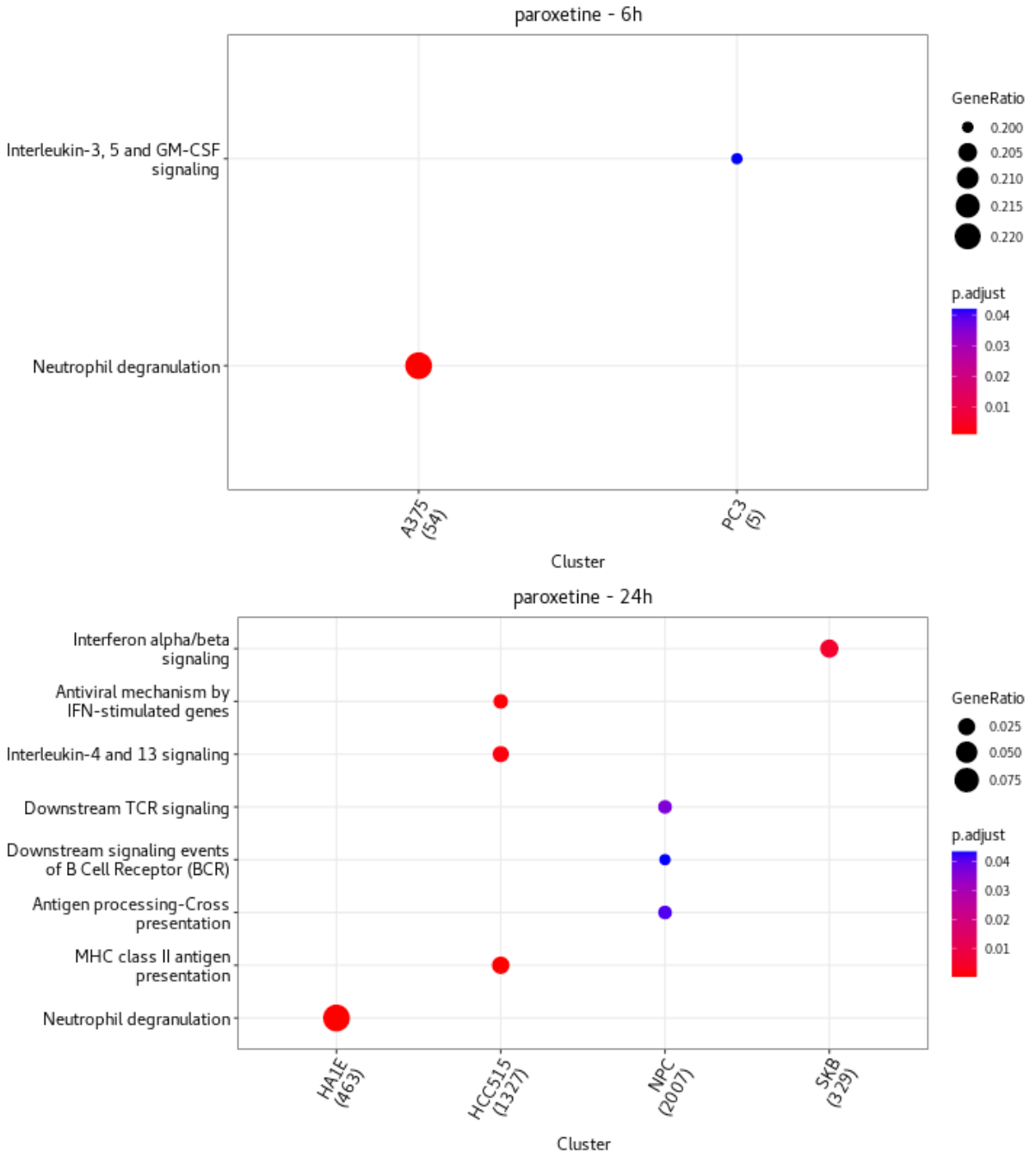

Figure F.14 – Functional enrichment analysis of the differential gene expression profile for antidepressant paroxetine using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

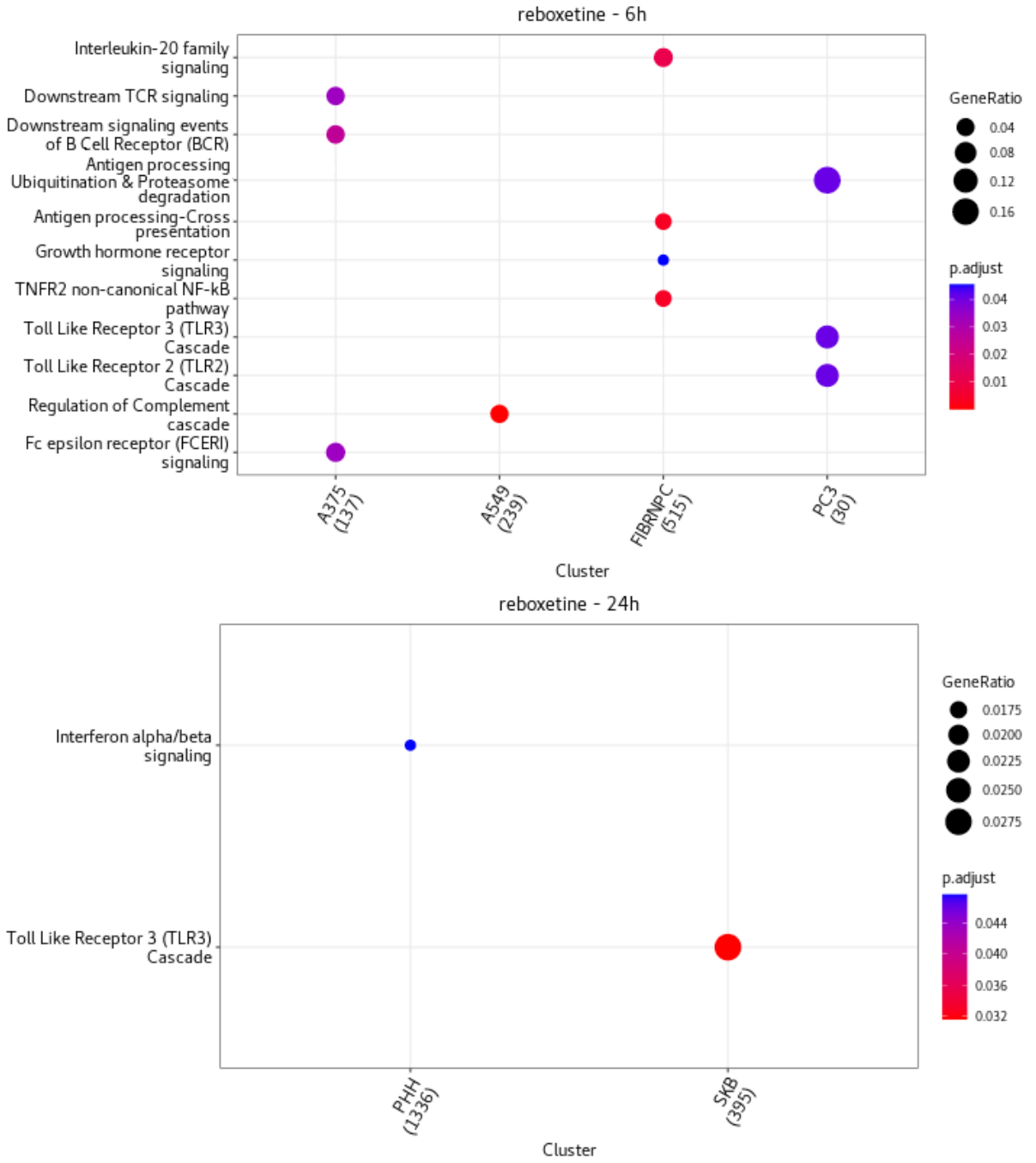

Figure F.15 – Functional enrichment analysis of the differential gene expression profile for antidepressant reboxetine using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

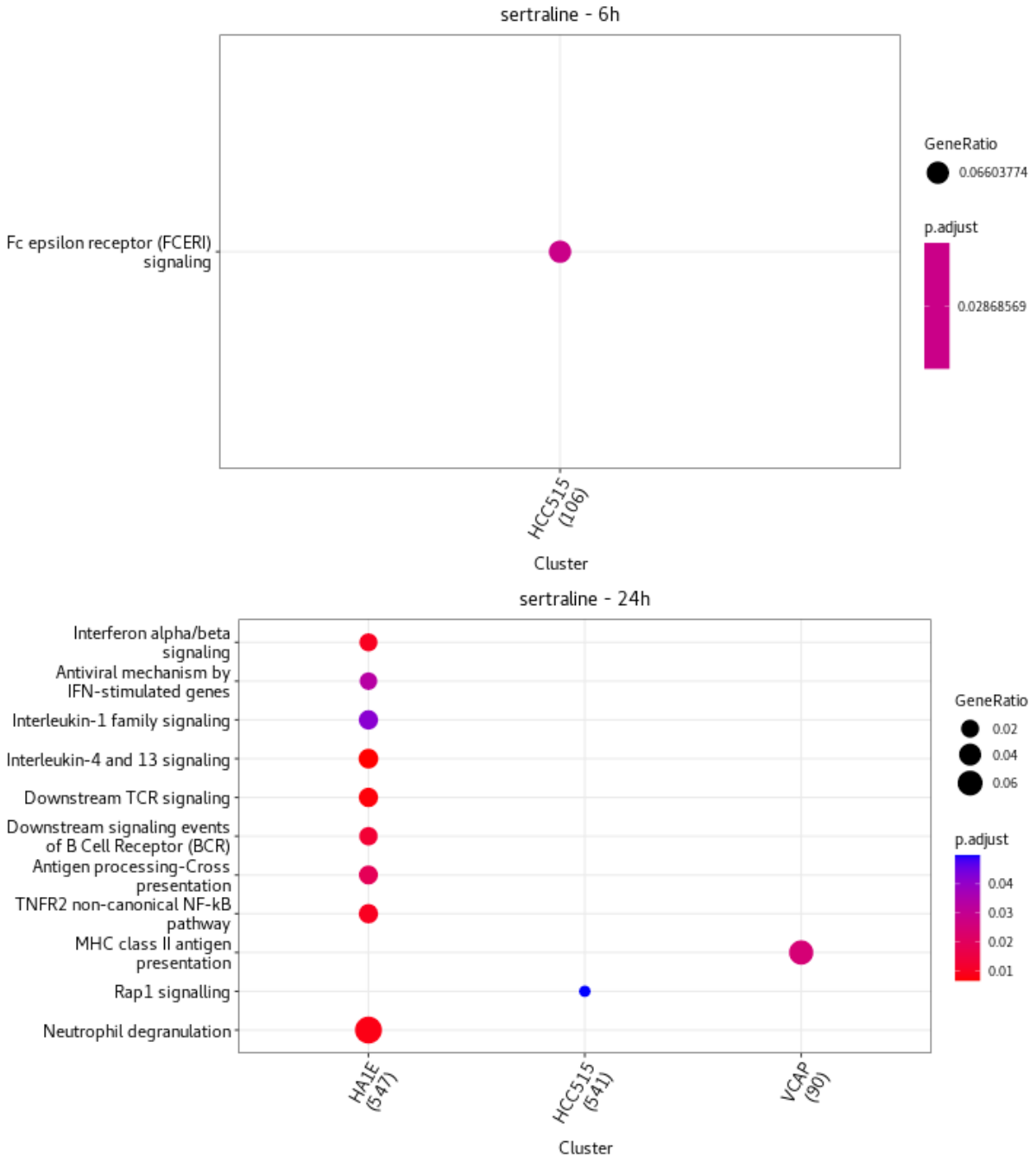

Figure F.16 – Functional enrichment analysis of the differential gene expression profile for antidepressant sertraline using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

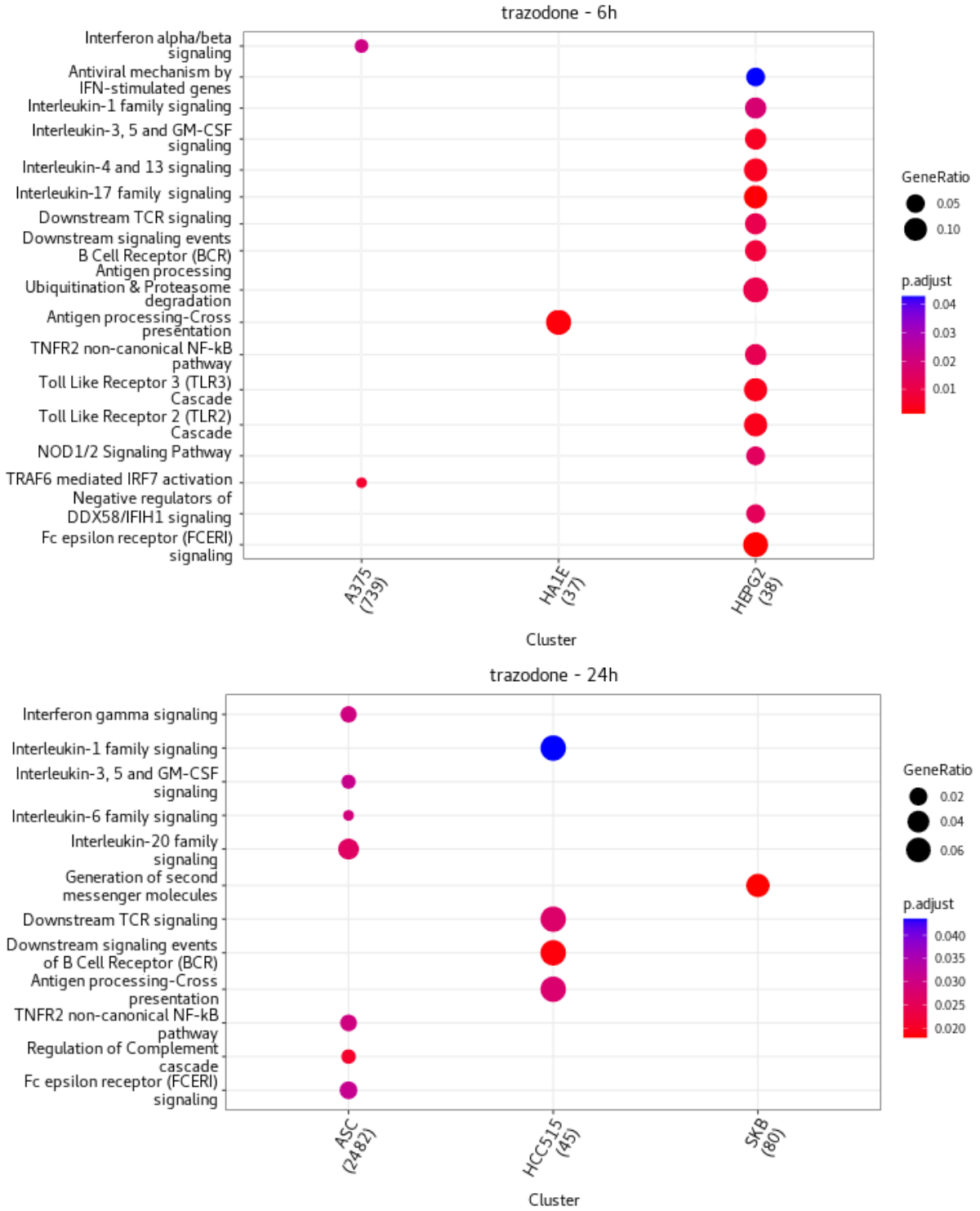

Figure F.17 – Functional enrichment analysis of the differential gene expression profile for antidepressant trazodone using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

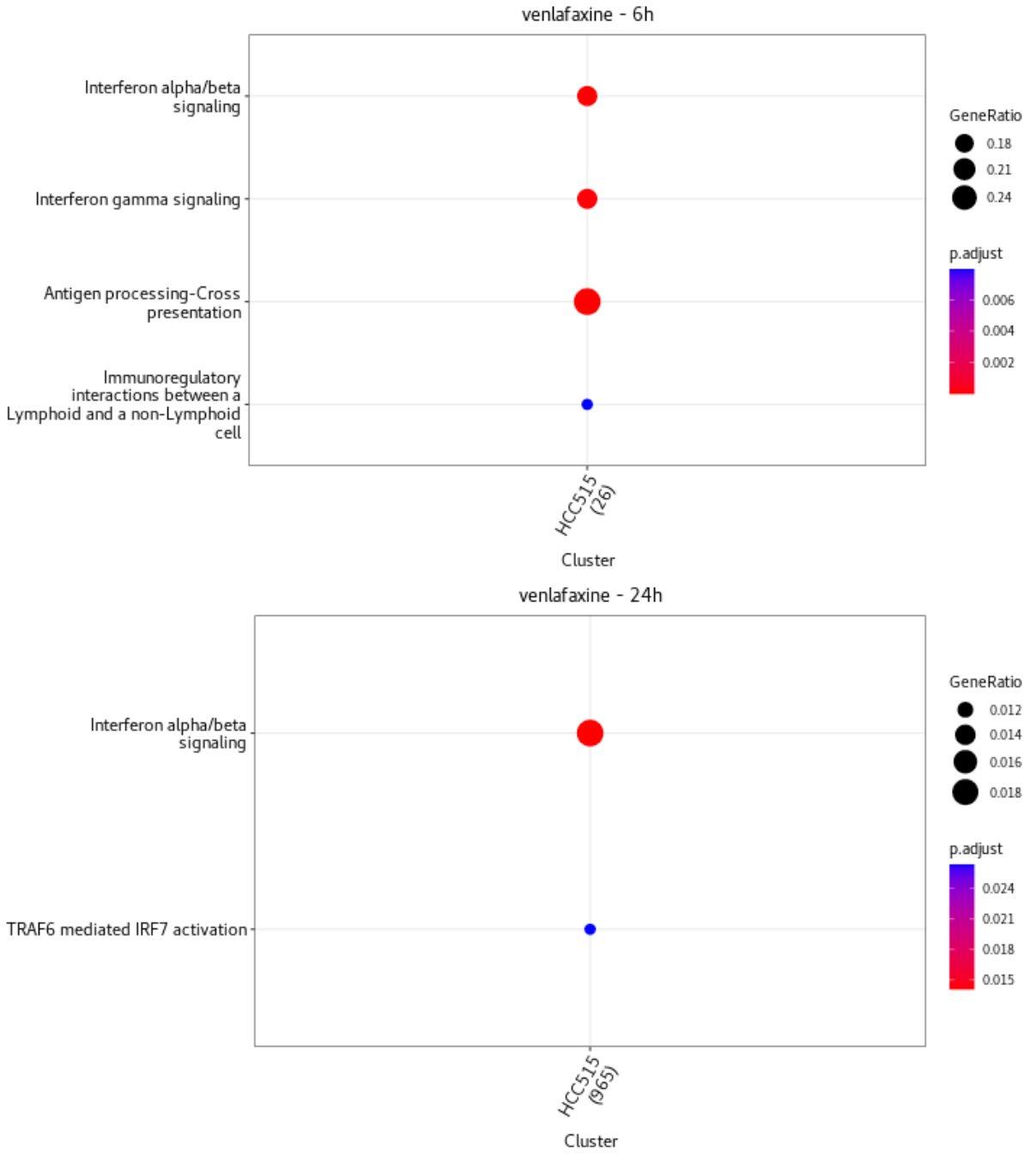

Figure F.18 – Functional enrichment analysis of the differential gene expression profile for antidepressant venlafaxine using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.

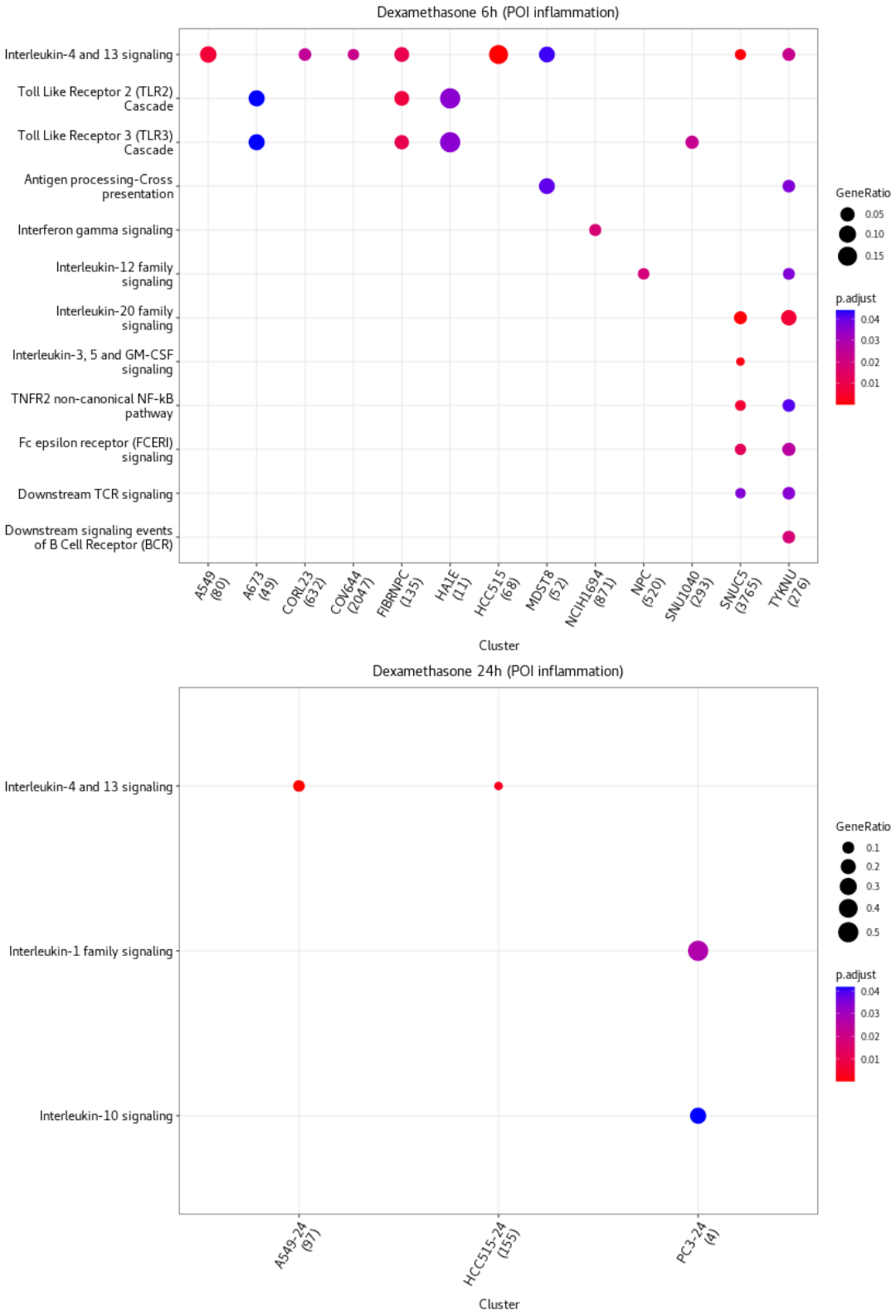

Figure F.19 – Functional enrichment analysis of the differential gene expression profile for glucocorticoid anti-inflammatory dexamethasone using Reactome (Fabregat et al., 2018). Cell lines were exposed for 6h and 24h. Only pathways related to the immune system have been selected.